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Effect of Advanced Age on the Innate Immune Response to Cutaneous Wound Infection

Aleah Lin Brubaker
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

EFFECT OF ADVANCED AGE ON THE INNATE IMMUNE RESPONSE TO CUTANEOUS WOUND INFECTION

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ALEAH BRUBAKER

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AMP	Antimicrobial peptide
APC	Antigen presenting cell
BSA	Bovine serum albumin
CFU	Colony forming unit
CRAMP	Cathelicidin related antimicrobial peptide
EpCAM	Epithelial cell adhesion molecule
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FcγR	*Fragment, crystallizable* gamma receptor
fMLP	formly-Methionyl-leucyl-phenyalanine
FoxO3a	Forkhead box O3a transcription factor
FSC	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H_2O_2	Hydrogen peroxide
hBD	Human β-defensin
HBSS	Hank's buffered saline solution
HSC	Hematopoietic stem cell
ICAM-1	Intercellular adhesion molecule-1

IL	Interleukin
IFN-γ	Interferon-gamma
iNOS	Inducibile nitric oxide synthase
i.p.	Intraperitoneal
LC	Langerhan's cell
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
mBD	Mouse β-defensin
MC	Mast cell
MCP-1	Monocyte chemoattractant protein-1
MFI	Mean fluorescent intensity
MIP1-α	Macrophage inflammatory protein-1α
MIP-2	Macrophage inflammatory protein-2
MLN	Mesenteric lymph node
MSA	Mannitol salt agar
MSC	Mesenchymal stem cell
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
NO	Nitric oxide
PAMP	Pathogen-associated molecular patterns
PAD	Peripheral arterial disease

PBS	Phosphate buffered saline
P. gingivalis	*Porphyromonas gingivalis*
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethanesulfonylfluoride
PLC	Phospholipase C
PRR	Pattern recognition receptor
PVA	Polyvinyl alcohol
Rac2	Ras-related C3 botulinum toxin substrate 2
Rap1	Ras-related protein-1
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
S. aureus	*Staphylococcus aureus*
s.c.	Subcutaneous
SEM	Standard error of the mean
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TAM	Tumor associated macrophage
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TREM-1	Triggering receptor expressed on myeloid cell-1
TSB	Tryptic soy broth
VCAM-1	Vascular cell adhesion molecule-1

VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4

ABSTRACT

An estimated 25 billion in US health care expenditure is spent on care of chronic, non-healing wounds. The failure to effectively heal wounds is often compounded by co-morbidities, such as diabetes or obesity. Another major patient population afflicted with chronic wounds are the elderly. Advanced age is associated with a decline in immunologic function that contributes to a poor response to vaccination, infection and tissue injury resulting in prolonged hospital stays and age-related morbidity and mortality. Specifically, clinical observations and laboratory studies have suggested an age-related decline in cutaneous wound healing, marked by protracted wound closure, wound dehiscence and chronic wound states.

Previous studies have suggested that age-related alterations in the early inflammatory phase of cutaneous wound injury may contribute to the lengthened and aberrant course of wound healing in the elderly. This impaired response to cutaneous injury offers a persistent portal of entry for foreign pathogens, such as *Staphylococcus aureus* (*S. aureus*), a common dermatopathogen that accounts for up to 50% of surgical site infections in the elderly. Underlying this elevated susceptibility to infection are deficits in host recognition, phagocytosis, migration and activation of a pathogen-specific adaptive immune response. Specific innate immune deficits have been tied to an elevated risk of *S. aureus* wound infection, including decreased neutrophil counts and function, reduced expression of cutaneous antimicrobial peptides and aberrant toll-like receptor 2 (TLR2)

expression and/or signaling. However, the effect of age on these specific alterations in the innate response has not been well described in a model of cutaneous wound infection. Thus, we hypothesized that advanced age contributes to a functionally inept innate immune response that impairs resolution of *S. aureus* cutaneous wound infection.

To elucidate the effects of advanced age on cutaneous wound infection, the specific aims were (i) To establish a model of local cutaneous wound infection in young and aged mice, (ii) To determine if age-related changes in leukocyte phagocytosis, leukocyte recruitment or cutaneous antimicrobial peptide expression contribute to age-associated differences in bacterial colonization and wound closure (iii)To determine if modulation of these age-associated deficits in the early innate response to *S. aureus* cutaneous wound infection may provide new therapeutic avenues for treating wound infection and promoting wound closure in the elderly.

Aim 1 was to establish of model of cutaneous injury and *S. aureus* infection in young (3-4 month) and aged (18-20 month) BALB/c mice. Following injury and infection at days 1-10, we observed heightened bacterial colonization and delayed wound closure in aged animals as compared to young.

In Aim 2, we utilized this model to evaluate keratinocyte and resident leukocyte TLR expression, leukocyte infiltration and phagocytosis and wound antimicrobial peptide expression. While no differences in infiltrating leukocyte TLR expression, phagocytosis or antimicrobial peptide expression were noted, decreased neutrophil and macrophage recruitment to the wound site were observed in aged mice relative to young. In an *in vivo*

chemotaxis assay, we confirmed a reduced migratory capacity to increasing doses of the neutrophil chemokine KC in aged mice.

Given these findings, we sought to modulate neutrophil recruitment to the wound site in Aim 3. Following cutaneous injury and infection, mice either received 3 *s.c.* injections of 250 pg of G-CSF or saline vehicle. G-CSF treatment in aged mice reduced bacterial colonization and wound size relative to saline controls. This was associated with increased neutrophil accumulation in wounds of aged, G-CSF treated mice at days 3 and 7 following injury and infection. While future work needs to be conducted to elucidate the mechanism by which G-CSF exerts these age-dependent effects, our data highlights a putative indication for G-CSF treatment within the wound care setting in the elderly patient population as well as patients with local or systemic neutropenia.

CHAPTER 1

INTRODUCTION

Advanced age is associated with a decline in immunologic function that contributes to a poor response to vaccination, infection and tissue injury resulting in prolonged hospital stays and age-related morbidity and mortality. Specifically, clinical observations and laboratory studies have reported an age-related decline in cutaneous wound healing, marked by protracted wound closure, wound dehiscence and chronic wound states. Previous studies have suggested that age-related alterations in the early inflammatory phase of cutaneous wound injury may contribute to the lengthened and aberrant course of wound healing in the elderly. This impaired response to cutaneous injury offers a persistent portal of entry for foreign pathogens, such as *Staphylococcus aureus* (*S. aureus*), a common dermatopathogen that accounts for up to 50% of surgical site infections in the elderly. Specific innate immune deficits have been tied to an elevated risk of *S. aureus* wound infection, including decreased neutrophil counts and function, reduced expression of cutaneous antimicrobial peptides and aberrant Toll-like receptor (TLR) 2 expression and/or signaling. However, the effect of age on these specific alterations in the innate response has not been well described in a model of cutaneous wound infection. Thus, we hypothesize that advanced age contributes to a functionally inept innate immune response that increases the susceptibility to cutaneous wound infection with *S. aureus*. To elucidate the effects of advanced age on cutaneous

wound infection, the specific aims of this proposal are (i) To establish a model of local cutaneous wound infection in young and aged mice, (ii) To determine if age-related changes in leukocyte phagocytosis, leukocyte recruitment or cutaneous antimicrobial expression contributes to age-associated differences in bacterial colonization and wound closure (iii)To determine if modulation of these age-associated deficits in the early innate response can enhance resolution of *S. aureus* wound infection in aged animals. Overall, this project will potentially reveal age-specific alterations in the innate immune response to cutaneous wound infection that may provide novel treatment options, ultimately improving clinical outcomes in the elderly.

Hypothesis: Impaired function of innate immune leukocytes and cutaneous antimicrobial peptides delays resolution of cutaneous wound infection with advanced age.

Aim 1: To establish a model of cutaneous *S. aureus* wound infection in young and aged mice.

Aim 2: To determine age-related alterations in innate immunity that may contribute to differences in bacterial colonization and wound closure with advance age.

Subaim 2A: To determine if an age-related impairment of phagocytosis and/or bactericidal activity promotes delayed resolution of wound infection in aged mice.

Subaim 2B: To evaluate leukocyte recruitment and factors involved in leukocyte infiltration that may contribute to differences in bacterial burden and wound closure in aged mice.

Subaim 2C: To examine if age-associated differences in cutaneous antimicrobial peptide expression contribute to heightened bacterial colonization and reduced wound healing in aged mice.

Aim 3: To determine if local, subcutaneous treatment with granulocyte-colony stimulating factor (G-CSF) can enhance resolution of cutaneous wound infection in aged mice.

CHAPTER 2

REVIEW OF RELATED LITERATURE

Aging, life expectancy and healthy living years

Health care advancements have contributed to a constant, linear increase in the expected lifespan in developed countries. In 1950, the probability of survival to 80-90 years of age was 12-16% and had doubled to 25-37% by 2002 (1). Interestingly, life expectancy data from the last century suggests that we have yet to reach the limits of human longevity (1), supporting the concept that the proportion of our population considered elderly, or over 65, will increasingly comprise a greater percentage of our population as a whole. Despite advances in health care that have improved human lifespan, our healthy living years are still limited by diseases that compromise activities of daily living and cognition. It has been reported that disease prevalence has grown in the aging population over time (1). While improved detection may account from some of the increase in disease prevalence, diabetes, obesity, cancer and heart disease have all risen markedly in the elderly. Of note, a dichotomy in the aging population is observed. Some individuals are able to maintain daily living activities, ward off infection and do not develop severe autoimmune or malignant conditions. Another sub-population, often referred to as the "frail" elderly, lose the ability to maintain clinical measures of independent living (i.e. management of medication, transportation, preparation of meal, etc.) and their physical deterioration is often marked by infection and significant pathology (1, 2). Given the seemingly unimpeded growth of the aging population, efforts to reduce chronic disease and improve healthy living years will greatly improve quality of life measures and curb the predictable strain on already unstable medical care systems.

A better understanding of the mechanisms that play a role in healthy and frail aging are critical to expanding healthy living years in the elderly and may help stifle a growing public health predicament.

Aging and Innate Immunity

A predominate contributing factor to these clinical ailments is dysfunction of the immune system. In particular, dysregulation of the innate immune response contributes directly to aging pathologies, as well as indirectly by altering activation of the adaptive arm of the immune response. In aged individuals, there is a paradoxical activation of baseline inflammation referred to as "inflamm-aging" coupled with a refractory response to immune challenge known as "immunosenescence" (reviewed in (3-6)). With advanced age, the inability to balance pro- and anti-inflammatory responses generates a basal elevation in pro-inflammatory mediators that may blunt appropriate innate and adaptive cellular immune activation and function (**Figure 1**). This disrupted balance between inflammation and immune activation may contribute to a variety of co-morbidities and increased mortality following both local and systemic insults (7-11). Herein, we discuss how aging negatively impact the host immune response and clinical outcomes by impairing three innate immune mediators: antimicrobial peptides, neutrophils and macrophages.

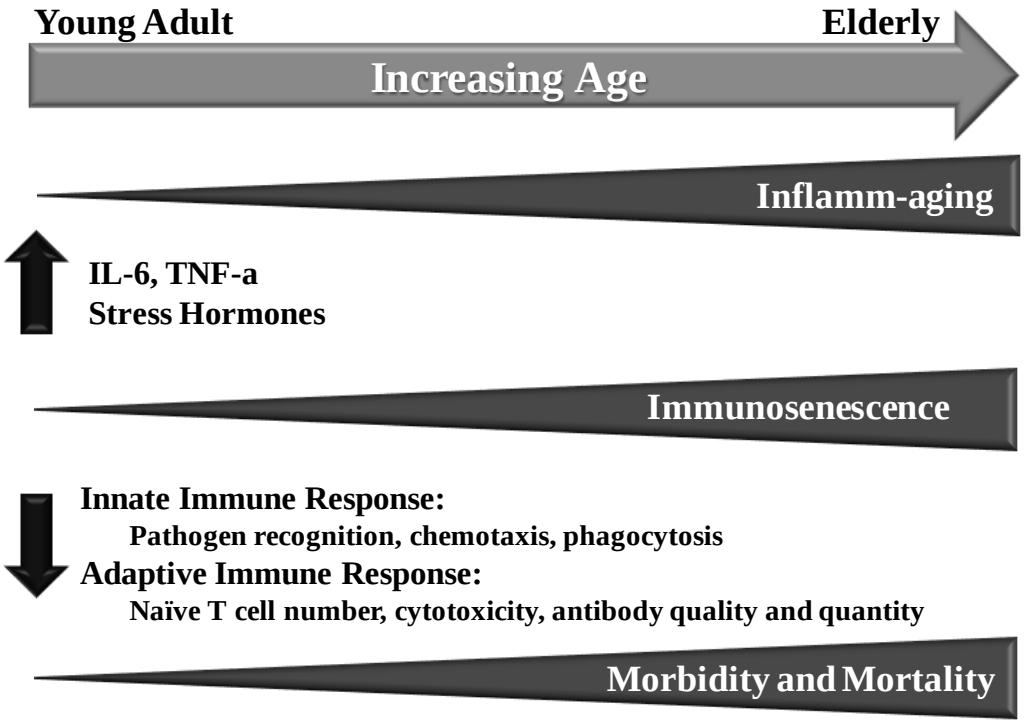


Figure 1. The skewed balance of the aging innate immune system. Advanced aged is marked by a heightened, basal pro-inflammatory response termed "inflamm-aging" that it built upon genetic, epigenetic and hormonal age-associated changes. Additionally, following antigenic challenge or systemic insult, immune cells from the elderly demonstrate an immunosenescent phenotype. Together, this results in greater morbidity and mortality following trauma or infection. Modified from (12).

Antimicrobial Peptides

Antimicrobial peptides (AMPs), such as defensins and cathelicidins, are small cationic molecules that are able to promote bacterial killing via interactions with the bacterial membrane, leading to membrane destabilization and bacterial lysis (13, 14). Several families of AMPs have been identified, including α-defensins, β-defensins, cathelicidins and the Reg family of AMPs. Over the past few years, these small molecules have garnered increasing attention for their bactericidal properties and modulation of wound healing (15-18). In addition to their bactericidal potential, AMPs are also known to be directly chemotatic for neutrophil and macrophages, highlighting their importance in regulation of the innate immune response (13, 19). However, very little has been published regarding the impact of advanced age on this innate immune subset. One study has reported that cathelicidin related antimicrobial peptide (CRAMP) mRNA expression was 10-100 fold higher in skin from neonatal mice as compared to adult mice, however aged animals were not examined (20). In another study, immunostaining for CRAMP and mouse β-defensin 3 (mBD3) in intact epidermis revealed reduced CRAMP levels in epidermal cells from 15-18 month aged mice as compared to young animals (21). However, levels of mBD3 in these aged animals were increased relative to young controls (21). Given the important immunomodulatory and host defense properties of these small molecules, further work to evaluate how they are impacted by age is warranted.

Neutrophils

Alongside AMPs, neutrophils are a critical first line of defense for the innate arm of the immune system in response to infectious challenge. Swift recruitment and activation of neutrophils occurs via integration of several, successive signaling steps involving selectins, integrins, chemokines and G protein coupled receptors (22, 23). The primary chemotatic signals for neutrophils in mice are KC and macrophage inflammatory protein-2 (MIP-2), both human interleukin (IL) -8 functional homologs. Other signals included complement factor C5a and leukotriene B_4. KC and MIP-2 interact with the neutrophil chemoattractant receptor CXCR2 to initiate intracellular signaling pathways involved in neutrophil chemotaxis (24, 25). Specifically, downstream of CXCR2, activation of the small GTPase Ras-related protein 1 (Rap1) has been show to be crucial in mediating adhesion by regulation of two integrins, lymphocyte-function associated antigen-1 (LFA-1: CD11a/CD18) and very late antigen-4 (VLA-4: CD49a/CD29; 26-28). Rap1-induction of integrin activity occurs via several convergent pathways downstream of CXCR2; however, the canonical pathway involves activation of phospholipase C (PLC; (26, 27, 29, 30). This upregulation of selectins and integrins promotes loose rolling followed by firm adhesion mediated by interactions with intracellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) present on activated endothelium. Firm adhesion coupled with apically suspended chemokines promotes neutrophil diapedesis to the site of injury or infection (**Figure 2**).

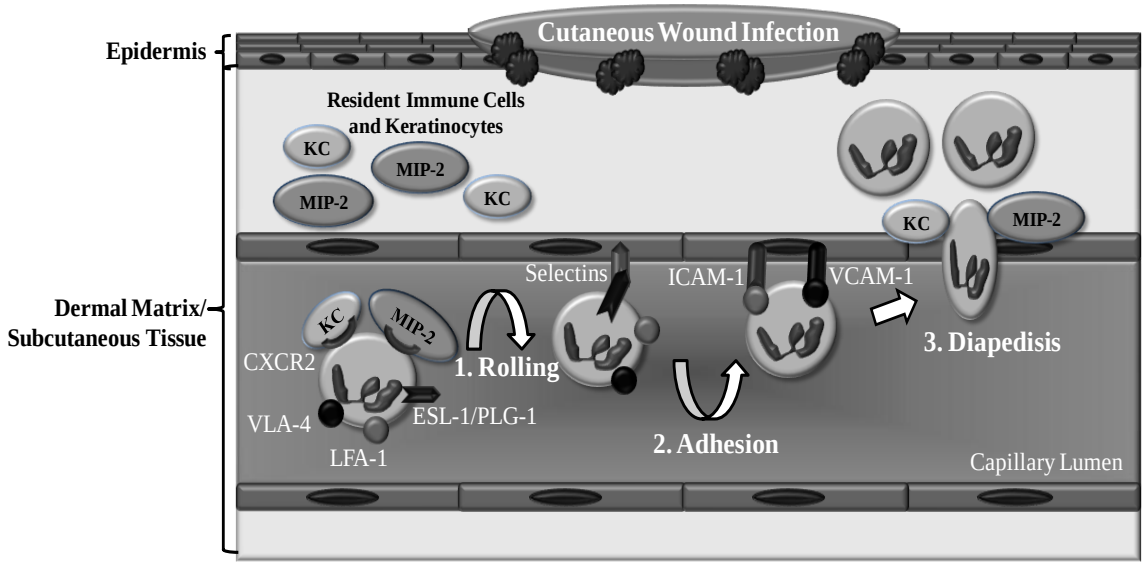


Figure 2. Neutrophil chemotaxis. Following cutaneous injury and infection, resident immune cells and keratinocytes release a host of soluble factors to initiate the innate immune response. In mice, release of the chemokines KC and MIP-2 act to recruit neutrophils by diffusing across endothelial cells to interact with their chemokine receptor, CXCR2, on peripheral blood neutrophils. Ligation of CXCR2 results in activation of PLC and Rap-1 to promote upregulation and clustering of selectins and integrins that help mediate (1) loose rolling followed by (2) firm adhesion via interaction with ICAM-1 and VCAM-1 on activated endothelium. In response to apically suspended chemokines, neutrophils can then undergo (3) diapedesis to the site of injury and infection.

Upon their recruitment, neutrophils act to clear invading microbes and debris by phagocytosis and intracellular killing mechanisms involving the generation of reactive oxygen species (ROS) and degranulate to release a variety of antimicrobial peptides, pro-inflammatory cytokines and hydrolytic enzymes (31-34)(35). Neutrophil granules contain multiple antimicrobial molecules and proteases including elastase, cathepsins, matrix metallopeptidase-9 and human neutrophil peptide-1 (34). Binding of pathogen-associated molecular patterns (PAMPs), such as formyl-Methionyl-Leucyl-Phenylalanine (fMLP), lipopolysaccharide (LPS) and other toll-like receptor (TLR) and triggering receptor expressed on myeloid cells-1 (TREM-1) ligands, to pattern recognition receptors (PRRs) promotes neutrophil activation. Cytokines including granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-15 and IL-18, can also prime neutrophils resulting in delayed or decreased apoptosis which subsequently lead to enhanced neutrophil effector functions (36-38). Activated neutrophils can then release cytokines and chemokines such as tumor necrosis factor-α (TNFα), IP-10, macrophage inflammatory protein-1α (MIP-1α), IL-12, KC, MIP-2, vascular endothelial growth factor (VEGF) among others to help drive the subsequent immune response (39).

In addition to pathogen recognition and inciting an inflammatory response, neutrophils play a critical role in clearance of invading pathogens. One mechanism for removal of foreign pathogens is the *Fragment, crystallizable* gamma receptor (FcγR)-mediated phagocytosis pathway (**Figure 3**). An invading organism is recognized by one of three FcγR (CD16, CD32 or CD64) and is internalized in a Ras-related C3 botulinum toxin substrate 2 (Rac2)-dependent manner. Activation of Rac2 , a small GTPase,

promotes interaction with cytosolic $p67^{phox}$ to form and recruit the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex to the maturing phagosome to mediate bactericidal functions (32, 40-42). Defects in the NADPH oxidase system, such as in chronic granulomatous disease, are associated with increased susceptibility and recurrent bacterial infections (43).

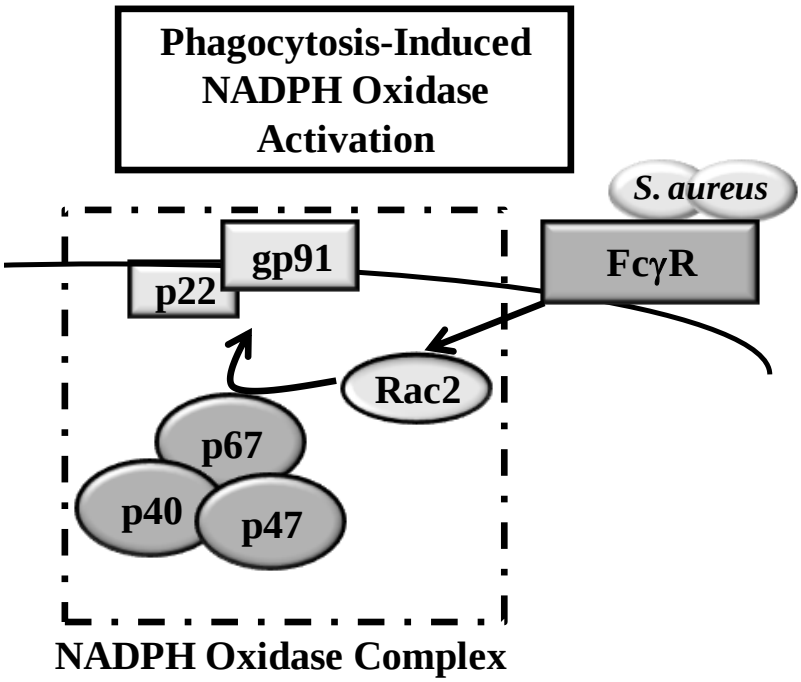


Figure 3. Neutrophil phagocytosis and assembly of the NADPH oxidase complex. Neutrophils play a critical role in clearance of foreign pathogen via FcγR-mediated phagocytosis. Following interaction between the FcγR and a pathogen, internalization and activation of the small GTPase Rac2 promotes interaction of the cytosolic $p67^{phox}$ complex with membrane bound p22 and gp91. Together, the NADPH complex is then guided to the maturing phagosome to help generate ROS and promote bactericidal activity.

Numerous studies in human and rodent models suggest that aging alters the circulating neutrophil pool and negatively impacts neutrophil effector functions, and that these changes can be amplified in the setting of trauma, infection and tissue injury (5, 44-52). With increased aged, there is a shift in the hematopoietic stem cell (HSC) pool towards generation of myeloid lineage cells, including neutrophils (reviewed in (53)). To extend these findings, Miyamoto *et al.* examined the impact of forkhead box O3a transcription factor (FoxO3a) in regulation of HSC differentiation in aged mice (54). The family of FoxO transcription factors mediate many cellular processes (55), and has been shown to be a player in HSC differentiation via modulation of oxidative stress (55). Considering the contribution of oxidative stress to immune dysregulation with age (reviewed in (56)), the impact of this particular pathway in the maintenance of the circulating neutrophil pool is of interest. Data from this group demonstrated that during recovery from myelosuppressive treatments, aged FoxO3a deficient mice exhibited profound neutrophilia compared to young knockout mice (54). This increase in neutrophils was associated with loss of inhibition of FoxO3a on Sprouty-related Ena/VASP homology 1 domain containing proteins 2 (Spred2), a negative regulator of cell proliferation, as well as activation of AKT and extracellular signal-related kinase (ERK) in the aged knockouts. This study provides a novel insight into maintenance of the HSC pool in aging, and extensions of these studies to examine the affect of age on expression of the FoxO family of transcription factors may further characterize the hematopoietic disruption and altered neutrophil development and function observed in aged animals.

Trauma and Tissue Injury

In the setting of systemic insults, such as those seen following traumatic injury or sepsis, an over-exuberant neutrophil response may contribute to the systemic immune response syndrome, and the associated morbidity and mortality in aging patients (57-59). Since the age of the patient is a major clinical predictor of outcome following trauma, several murine studies have investigated how neutrophils as early immune mediators may be contributing to poor clinical outcomes after major trauma and tissue injury (11, 48-50). Our laboratory has previously shown that aged mice exhibit increased mortality following a 15% total body surface area full-thickness burn trauma as compared to young mice subjected to the same injury (11). These aged mice have elevated levels of the neutrophil chemokine KC in lung tissue which translated into heightened neutrophil infiltration and pulmonary inflammation 24 hours following burn injury (48). Administration of anti-CXCR2 antibody in aged mice reduced levels of neutrophil accumulation and pulmonary inflammation to levels observed in young mice (48), perhaps suggesting that modulating the neutrophil response following systemic insult may decrease pulmonary co-morbidities. Extending these studies, we observed that expression of endothelial ICAM-1 was elevated on the pulmonary endothelial cells following burn injury in aged mice as compared to young, suggesting that the age-related changes in the pulmonary vasculature that wound impact neutrophil extravasation with advanced age (60). These data are supported by reports that neutrophils from elderly donors demonstrated increased adherence to an endothelial cell monolayer following fMLP or phorbol 12-myristate 13-acetate (PMA) stimulation (61). However, two other studies determined that neutrophil adhesion to endothelial cells, expression of adhesion

molecules and neutrophil recruitment were comparable between young and aged donors (46, 62). These studies highlight differences not only between human and murine models, but also highlight how aging can exacerbate differences between neutrophil responses prior to and post injury.

In the setting of local tissue injury, alterations in neutrophil infiltration may also play a role in delayed resolution with advanced age. Following cutaneous tissue injury, neutrophils are the first phagocyte to enter the wound bed, helping to incite an inflammatory response against invading organisms. In aged BALB/c mice, Swift *et al.* observed a significant delay in wound closure as reported in other studies (49, 50, 63, 64). Though age-related differences in neutrophil recruitment to the site of cutaneous injury were observed at early time points, they did not reach significance (50). Conversusly, Nishio *et al.* demonstrated that neutrophil peak infiltration was delayed in aged C57BL/6 mice following cutaneous injury (49). Moreover, Gr-1 mediated neutrophil depletion further delayed wound closure in aged mice. Systemic administration of G-CSF or topical application of peritoneal-derived neutrophils from young mice restored rates of wound closure to those observed in young mice (49). This reduced neutrophil chemotaxis with advanced age is recapitulated in *ex vivo* murine and human studies. In BALB/c mice, we recently reported that neutrophils from aged mice demonstrate a hyperchemokinesis in the absence of a neutrophil chemotatic stimulus; however, in the presence of increasing doses of KC, neutrophils from aged mice demonstrate a marginal chemotatic response as compare to neutrophils from young mice (60). Similarly, peripheral blood neutrophils isolated from healthy, aged subjects

conforming to the SENIEUR protocol demonstrated reduced migration towards fMLP and GM-CSF (45).

Though fetal or adult wound healing studies often consider neutrophil recruitment detrimental to wound closure and scar formation (65), these studies highlight how findings in young model organisms are not always directly applicable to the aged organism. Moreover, the differing results in these between human and rodent studies reflect a major caveat in aging research. Namely, contradictory results between human and rodent studies can make it difficult to draw parallels between these studies, and future animal models need to closely mimic clinically relevant scenarios to improve the correlation between these data sets. Additionally, different animal strains can lead to alternative outcomes, further complicating rodent research models. Future *in vivo* rodent models and injury paradigms should be evaluated to determine their relative correlation with findings in human studies to optimize rodent aging research. Though additional studies need to be conducted to better establish the role of neutrophils in wound healing and trauma, the impact of advanced age on neutrophil infiltration kinetics and function may contribute to adversus clinical outcomes such as suboptimal healing and infectious complications in the elderly.

Infection and Environmental Exposures

Neutrophils are critical for effective recognition and removal of debris and foreign pathogens. A major pathway that aids in recognition of non-native particles is the TLR pathway. In humans, aging does not impact basal levels of TLR2 and TLR4 expression on neutrophils (45). However, aging does increase neutrophil TLR4

expression in unstimulated lipid rafts and non-raft fractions compared to the young mice. Stimulation with the TLR4 ligand LPS markedly induced TLR4 recruitment to lipid rafts in young mice; however, aged mice did not alter recruitment or redistribution of TLRs between lipid raft and non-raft fractions (45). Expression of MyD88, a common downstream signaling molecule of several TLRs, on neutrophils isolated form young and aged mice was comparable after LPS stimulation. Interestingly, in the same study, parallel work in human neutrophils demonstrated reduced membrane-associated MyD88 in neutrophils from aged subjects after stimulation (45).

Similar to difference in membrane/raft-associated TLRs and MyD88, TREM-1 localization to lipid rafts was decreased with age following exogenous stimulation and this correlated with reduced TREM-1-induced neutrophil functions including degranulation, phagocytosis, ROS production and production of pro-inflammatory cytokines and chemokines (66). Of note, elevated circulating levels of soluble TREM-1 are associated with heightened inflammation as well as morbidity and mortality in critically ill patients with sepsis (67). In addition to TREM-1, Fc receptors play a predominate role in neutrophil phagocytosis and ROS generation. Studies in neutrophils isolated from healthy, elderly subjects demonstrate decreased FcγRIII expression (CD16), and this correlated with reduced phagocytic potential. Together, these data suggest a potential for reduced pathogen recognition, phagocytosis and bactericidal potential with age that may negatively impact outcomes following bacterial or viral infection in aging subjects.

The studies mention above primarily focused on *ex vivo* stimulation of neutrophils to generate age-dependent changes in neutrophil effector functions. To extend these studies, others have examined the impact of age on neutrophil function in *in vivo* models of infectious challenge or environmental exposures (51, 52, 68-70). In one such study in aged mice, exacerbated LPS-induced pulmonary inflammation was correlated with increased lung levels of KC, MIP-2, IL-1β and prolonged pulmonary neutrophilia at 72 hours (70). In another model of pulmonary challenge, intranasal infection with *Francisella tularensis* lead to an altered lung inflammatory response, with a delay in production of neutrophil chemokines MIP-2 and CXCL6 and attenuated neutrophil infiltration into the lung tissue in aged mice (51). Moreover, environmental insults, such as air pollutants or cigarette smoke, contribute to airway inflammation and chronic pulmonary co-morbidities (71, 72). Aged mice subjected to inhaled diesel exhaust demonstrate enhanced pulmonary congestion and neutrophilia 24 hours after exposure as compared to young mice (52). Similarly, single or chronic cigarette smoke exposure in aged mice resulted in elevated levels of KC and MIP-2, and prolonged neutrophil recruitment to the lung (73). This age-related increase in cytokine production was associated with amplified nuclear factor (NF)-κB expression and nuclear translocation (73). Looking at these findings, in response to pulmonary challenge, aged mice have an elevated and/or prolonged neutrophil response. Considering the delicate lung alveolar architecture and the highly hydrolytic enzymatic degranulation products of activated neutrophils, this may contribute to excessive tissue damage and reduced lung function over time.

The role of neutrophils in other models of infection have also garnered interesting data. Following oral infection with *Salmonella Typhimurium*, aged mice have elevated bacterial colonization in the ileum, colon, Peyer's patches, mesenteric lymph nodes (MLN) and liver as compared to young mice (68). Despite a baseline elevation in neutrophil numbers in the spleen and MLN of aged mice, aged mice did not increase neutrophil numbers in response to infection whereas young mice mounted a more significant neutrophil response (68). Following challenge with the fungal pathogen *Candida albicans*, decreases in neutrophil recruitment to the peritoneal cavity were associated with reduced fungicidal activity in aged mice (74), again highlighting how neutrophil recruitment may be dysregulated with advanced age.

Combined, these studies offer significant evidence suggesting that neutrophil recognition, phagocytic potential and infiltration kinetics are dysregulated with aging. This dysfunction may have pleiotropic effects on the host depending on the inciting injury or clinical paradigm by promoting pathogen dissemination, compromising organ function and delaying time to recovery. While altered neutrophil pathogen recognition, infiltration and phagocytosis may impair the ability of the innate immune response to contain microbes to site of origin, prolonged neutrophilia can result in significant tissue damage and inflammation via potent neutrophil hydrolytic enzymes, demonstrating the importance in precise temporal regulation of this particular immune cell subset.

Macrophages

Macrophages play pivotal roles in modulating immune and stromal cell function following infection, tissue injury and in tumor cytotoxity (75-77). Following extravasation from the peripheral blood pool, monocytes undergo maturation into tissue

macrophages. As professional antigen presenting cells, macrophages help shape the innate and adaptive immune responses through the release of a variety of pro-inflammatory mediators at early time points following injury or infection. Collaboration of macrophages with T and B cells is further mediated by the release of cytokines, chemokines, enzymes, arachidonic acid metabolites and reactive radicals (78, 79). Macrophages also serve to dampen the inflammatory response through phagocytosis of apoptotic cells and secretion of soluble factors like IL-10 (80). Age-related alterations in macrophage number and function have been correlated with enhanced susceptibility to infection, differential responses to tissue injury and enhanced tumor progression in rodent models (74, 81-83).

Macrophage Population Dynamics

With age, there is some discrepancy regarding changes in innate and adaptive immune cell numbers in rodent and human studies (68, 83, 84). In circulation, the number of blood monocytes in aged and young subjects appears to be comparable; however, there is a significant decrease in macrophage precursors as well as macrophages in the bone marrow of the elderly (85, 86). This may be related to a longitudinal decrease in macrophage bone marrow production over the human lifespan as a decrease in the percentage of cells expressing the $CD68^{+}$ cells, a macrophage marker, was observed in bone marrow from adults as compared to children (86). In murine studies, one report suggests that there is an increase in the Mac1/CD11b population in aged murine bone marrow (87), though others have found there were no differences in the number or DNA content of developing macrophages between young and aged mice (84). In other cellular

compartments, aged mice have been reported to have increased resident macrophages in the spleen (68), though there is a reduction in the marginal zone macrophage population and a breakdown of the marginal zone architecture with advanced age (83). This collapse in marginal zone structure may impair clearance of circulating pathogens and follicular responses in the elderly, increasing susceptibility to infection and prolonging recovery. Though human and rodent studies have yet to generate a conclusive answer regarding macrophage numbers in various compartments, alterations in macrophage environmental distribution may generate organ specific dysregulation. For example, increased infiltration of macrophages into adipose tissue of aged C57/BL6 mice fed high caloric diets was associated with increased inflammasome activation and cytokine production (88). These data suggest that a tissue-specific shift in macrophage populations can contribute to basal differences in the inflammatory profile of aged individuals.

Cytokine Production and TLR Signaling

In rodent models, advance aged is associate with increased levels of circulating cytokines, in particular IL-6, and it has been speculated that changes in these pro-inflammatory mediators may alter immune function in aged animals (11, 89-91). Despite this basal elevation, challenge of bone-marrow derived macrophages with *Porphyromonas gingivalis (P. gingivalis)* produce significantly less tumor necrosis factor-α (TNF-α) and IL-6 in aged mice (92). The influence of aging on LPS-mediated cytokine production selectively impact macrophage responses, such that some functions are depressed while others are elevated. For example, macrophages from aged mice

have increased levels of cyclooxygenase-2 and produced elevated levels of prostaglandin E_2 and IL-10 after LPS stimulation (93-95), whereas TNFα, IL-6 and IL-1 and IL-12 production are decreased as mentioned above. Similarly, in response to LPS, splenic macrophages from aged wild type mice demonstrated diminished responsiveness to LPS stimulation, producing lower levels of TNF-α, IL-1β, IL-6 and IL-12 as compared to young controls (96). Interestingly, cytokine production was restored following *in vitro* culture of LPS with splenic macrophages from aged IL-6 deficient mice (96), suggesting that elevated systemic IL-6 may directly impair macrophage function in aged animals. Several studies also support a similar reduction in cytokine production, including TNF-α, IL-6, IFN-γ, nitric oxide (NO), monocyte chemoattractant protein-1 (MCP-1) and MIP-1α, following a variety of stimuli (68, 74, 92, 97).

Generation of cytokines is often examined following stimulation of a variety of TLRs. TLRs help initiate the innate immune response to a variety PAMPs, and signal through either MyD88-dependent or –independent pathways to promote NF-κB pro-inflammatory cytokine production. Reduced expression of these receptors, or alterations in their respective downstream pathways, can increase susceptibility to infection (98-100), and a number of studies demonstrate alterations in the macrophage TLR expression and/or signaling in aged animals and humans. In both splenic and peritoneal macrophages of aged C57/BL6 mice, decreased expression in all TLRs (TLR1-9) have been found (74, 101), though other studies report no difference in expression of some TLRs, including TLR2 and TLR4, in aged BALB/c (82, 102, 103).

A study of 159 young and aged individuals found an age-associated reduction in TNFα and IL-6 by peripheral blood monocytes after stimulation with TLR1/2 that correlated with reduced surface expression of TLR1 (104). An age-associated decrease in TLR4 surface expression and and TLR7-induced IL-6 production was also observed in this study (104). Recent research provided similar evidence for age-associated defects in TLR function in human macrophages in the context of infection with West Nile virus (99).

While divergent results regarding TLR expression may be due to purity of the isolated macrophage population or murine background strain, the pathways downstream of TLRs may also be impaired with age. In one such study, LPS or zymosan induced TNF-α and IL-6 production was attenuated in splenic macrophages from aged mice due to reduced activation of p38 mitogen-activated protein kinase (MAPK), MAPK-activated protein kinase-2 and NF-κB (102). Others have shown that thioglycollate-elicited peritoneal macrophages from aged mice exposed to *P. gingivalis* have increased gene expression of the single immunoglobulin interleukin-1-related receptor (SIGIRR) which acts to inhibit TLR MyD88-dependent signaling (103, 105). In aged rodent models, downregulation of other members of these pathways, like MyD88 and tumor necrosis factor receptor-associated factor 6 (TRAF6), lead to decreased activation of the NF-κB family of transcription factors (69, 106). While studies have shown that age does not affect the basal levels of LPS-binding protein (96), levels of CD14, a co-receptor for TLR2 and TLR4, were reduced in macrophages from aged mice (107). Downregulation of mediators of the TLR pathways would dampen the immune

response, and may account for the decrease in cytokine production seen in aged rodent and human macrophages following immunogenic challenge.

ROS/RNI Generation and Macrophage Phenotype

Another major mediator of macrophage effector function is the generation of ROS and reactive nitrogen intermediates (RNI) that aid in intracellular microbicidal activity and tumor cytotoxicity. ROS and RNI, alongside IL-1, IL-12, TNF-α, represent the pro-inflammatory M1 macrophage profile, and these cells function in driving Th1 responses, resisting tumor development and killing of intracellular foreign pathogens (80). On the other hand, M2, or anti-inflammatory, macrophages have elevated levels of IL-10 and arginase expression. M2 macrophages predominately function in immunoregulation, neovascularization, tissue remodeling and tumor growth (80, 108). Macrophages from aged mice had been shown to produce elevated levels of IL-10 and reduced FasL, IL-12 and TNF-α, mediators that contribute to an M2 anti-inflammatory profile (109). Several studies have also reported decreases in ROS or RNI generation with age that may represent a skewing of M1 and M2 polarization with age (81, 110, 111). As compared to young animals, alveolar macrophages from senescent rats demonstrated a decreased baseline generation of ROS and NO, and LPS treatment did not significantly change ROS and NO production in aged animals (110). In response to PMA or zymosan, peritoneal macrophages from aged mice produce 50% less hydrogen peroxide (H_2O_2) in contrast to cells from young animals (111). These studies are supported by previous reports in which NO production by splenic or peritoneal

macrophages was reduced in several mouse strains following LPS or peptidoglycan-polysaccharide (112). In addition to these findings, macrophages from aged mice exhibit reduced GM-CSF mediated cell proliferation following exposure to H_2O_2 due to diminished signal transducer and activator of transcription (STAT) 5a oxidation and phosphorylation, suggesting that aging increases macrophage susceptibility to oxidative stress (113).

NO production is modulated by enzymatic activity of inducible nitric oxide synthase (iNOS) via activation of the interferon-gamma (IFN-γ) receptor. In aged BALB/c mice, mRNA expression of iNOS was found to be decreased after stimulation with LPS as compared to young mice (112). Similarly, our laboratory has observed decreased iNOS expression in splenic adherent cells from aged BALB/c mice following stimulation with IFN-γ and TNF-α (114). As macrophage signaling through the IFN-γ receptor promotes ROS and RNI generation, several studies examined if alterations in this receptor and the downstream signaling pathway may exist with advanced age (111, 115, 116). Though there are no differences in IFN-γ receptor with age (111, 115), culture with IFN-γ reduced H_2O_2 and NO_2^- production in aged animals due to decreased phosphorylation of MAPK (111). Others also found decreased STAT1a gene and protein expression and reduced STAT1 phosphorylation after *in vitro* IFN-γ stimulation of macrophages isolated from aged BALB/c mice (115). While this reduction in iNOS and NO production may suggest suppression of M1 macrophages with age, our lab has also observed a reduction in arginase-1 expression after IL-4 stimulation in adherent splenocytes from aged mice (114), implying a global immunosuppression of

macrophage function with age. Together, these studies suggest that the ROS and RNI pathways are dysregulated with age, translating into potential differences in macrophage phenotype and intracellular killing capacity, and ultimately negatively impacting immune function in the elderly.

Contrary to these reports, Chen *et al.* found enhanced NO production in both thioglycollate-elicited and resident peritoneal macrophages from inbred CBA/CA aged mice following culture with LPS, zymosan or heat-killed *S. aureus* (117). In these animals, elevated NO production was correlated with a persistent increase in iNOS expression. Yet another study found no difference in iNOS induction, NO production or intracellular killing in thioglycollate-elicited macrophages from aged BALB/cByJ mice in response to *in vitro* stimulation with *P. gingivalis* (103). These studies highlight that a variety of factors, including the murine strain, macrophage population and inciting stimuli, all play are role in determining potential age-related differences in macrophage function in rodent models.

Tissue Injury

As temporal changes in macrophage phenotype have been implicated in wound resolution, alteration in macrophage phenotype and function in aged animals may provide insight into altered tissue repair with advanced age (50, 64, 118). In particular, in murine models of tissue injury, aged animals have a prolonged course of wound repair, associated with delays in re-epithelialization, neovascularization and restoration of the extracellular matrix (63). Following excisional wound injury, macrophage accumulation was elevated 56% at day 1 in aged mice as compared to young, and this

correlated with increased MCP-1 production (50). In macrophages isolated from subcutaneous implanted polyvinyl alcohol (PVA) sponges, aged mice demonstrated an approximate 40% decrease in phagocytosis as compared to young mice (50). These delays in wound closure and diminished phagocytic ability of wound cells may contribute to the increased susceptibility to infection seen in elderly patients (119-121). Additionally, reduced macrophage phagocytosis may impair the ability to clear apoptotic keratinocytes or neutrophils from the wound bed, further delaying wound repair. Aprahamian *et al.* recently demonstrated that following ultraviolet B irradiation, aged mice demonstrated an elevated percentage of apoptotic keratinocytes compared young irradiate mice that was later correlated with reduced phagocytic capacity and apoptotic cell clearance in aged animals as compared to young (122).

As macrophages act as to bridge the innate and adaptive immune responses, an interesting study by Agius *et al*. recently demonstrated that cutaneous delayed-type hypersensitivity responses to bacterial, fungal or viral antigens by $CD4^+$memory T cells is significantly decreased in older subjects (123). The defective T cell immunosurveillance was attributed to diminished production of TNF-α by cutaneous macrophages, resulting in reduced endothelial activation and a subsequent decrease in T cell recruitment to the aging skin. This study emphasize how a defect in the macrophage population with advanced age not only impairs the innate arm of the immune system, but can compromise the pathogen-specific adaptive immune response and result in a poor prognosis following injury and infection. Together, these findings may provide the foundation for improving our understanding of poor clinical outcomes

following tissue injury and infection with aging, and may lead to design of therapeutic interventions to enhance macrophage function and accelerate wound closure.

Tumor Immunity

Deficits in macrophage function also directly contribute to the increased incidence of neoplastic growth and metastasis in elderly patients. In tumor immunology, recruitment of tumor associated macrophages (TAMs) results in the release of growth factors, proteases and inflammatory mediators, promoting vascularization of tumor tissue and metastasis (124). Infiltration of tumors by TAMs is associated with a poor prognosis (124), and age-related differences in TAM effector functions within tumors have been identified (125). In a B16 intraocular tumor model, aged mice exhibit increased infiltration of M2-polarized macrophages in contrast to young mice. These macrophages had elevated expression of pro-angiogenic VEGF and tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (TIE-2) (125). Depletion of macrophages by subconjunctival injections of clodronate-containing liposomes reduced tumor growth in 86% of mice compared to only a 10% reduction in young animals. Interestingly, the M2 macrophages in tumors from aged mice shared some phenotypic characteristics of myeloid-derived suppressor cells from the monocytic lineage, namely Ly6-C (125) and were also subject to depletion by the methods used above. In addition to TAM recruitment, macrophages from aged rodents have elevated cycloxygenase activity and prostaglandin E_2 synthesis, mediators that have been recently implicated in tumorigenesis (93, 126). Further work to better characterize TAMs with aging and cancer subtypes may aid in development of targeted

cancer treatments for patients with poor prognoses due to TAM infiltration and subsequent neovascularization and metastasis.

Age-related differences in macrophage function described above highlight how alterations in macrophage phenotype and function can facilitate development of infection, delays in tissue repair and impairment of tumor regression. Utilizing targeted rodent models to increase our understanding of how macrophages can contribute to specific pathological conditions may position us to better understand disease states at the bedside.

Summary of Aging and the Innate Immune System

Age-associated impairment in the various components of the immune system has been extensively studied. However, it is evident from the recent studies reported herein that some aspects of innate immunity require more investigation than others (e.g. AMPs). Tackling immunosenescence as it affects the innate arms of the immune system is a logical step forward as efficient antigen presentation and co-stimulation by macrophages and dendritic cells may promote a 'normal' humoral and cell-mediated immune response in aged individuals. Moreover, future aging research should focus on the key signaling molecules modulating the various signaling pathways involved in phagocyte and antigen presenting cell (APC) effector responses as well as cross-talk between these pathways. Furthermore, studying the intrinsic defects of a cell without the interference of the aged microenvironment is as crucial as focusing on the overall aged phenotype. Tailored and complementary i*n vitro* and *in vivo* studies on the complex physiological interactions

between innate immune cells and tissue-specific environments can hold a promising future for aging research.

Innate Immune Cells as Regulators of Wound Healing

Cutaneous wound healing is a dynamic process requiring coordination of immune cell infiltration, keratinocyte proliferation, angiogenesis, extracellular matrix deposition and collagen remodeling to ensure adequate wound closure (127-129). Proper regulation of this physiologic response to injury by crosstalk between various cell types and soluble factors facilitates restoration of the epidermal and dermal barrier with minimal scar formation (127, 130, 131). Multiple pathologic conditions, hormone status and advanced age are known to perturb efficient cutaneous repair by impacting one of the three interdependent phases of wound healing: the early inflammatory phase, proliferative phase or late remodeling phase (**Figure 4**) (127, 128, 131, 132). Innate immune cells are critical during these stages of wound repair, as changes in innate immune cell function have been shown to significantly augment normal wound healing, leading to poor wound closure or hypertrophic scar formation. Understanding the contribution of the innate immune response to wound repair and dermal restoration is instrumental in the rational development of clinical therapeutics that will enhance wound closure, decrease associated morbidities and reduce critical care costs.

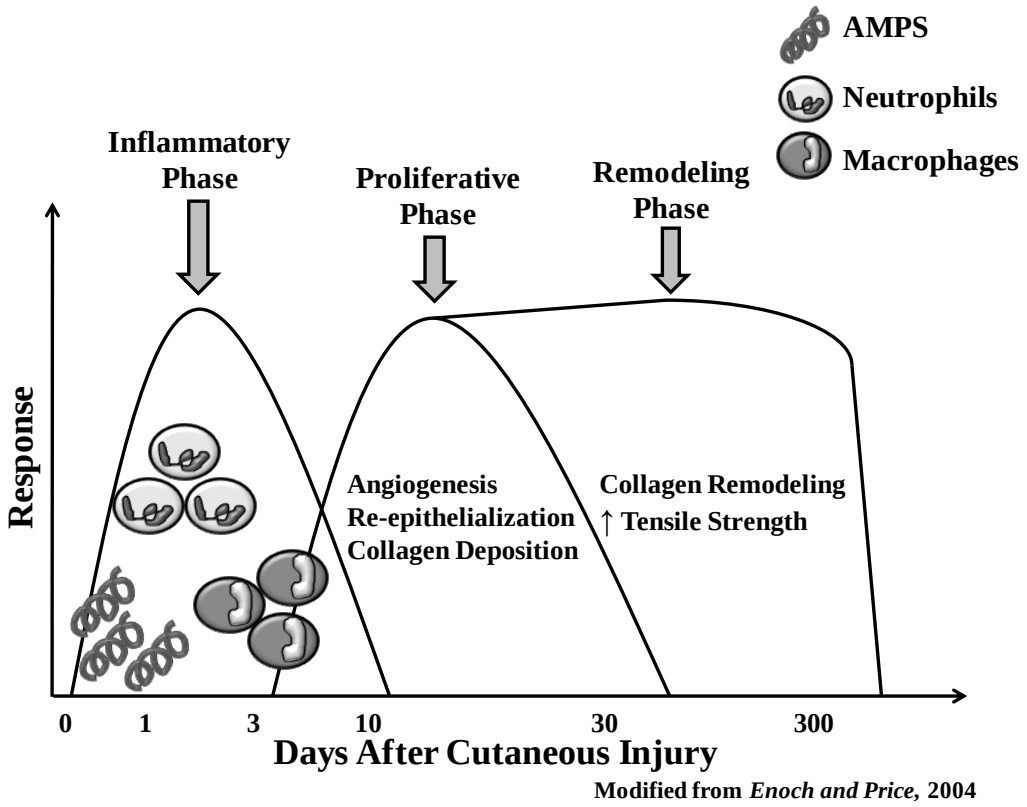


Figure 4. Phase of cutaneous wound healing. *Inflammatory phase*: Immediately following tissue injury, platelets, local immune cells and keratinocytes release soluble mediators such as AMPs and pro-inflammatory cytokines/chemokines to help kill invading pathogens and recruit innate leukocytes. Then, neutrophil infiltration occurs as early as 6 hours and peaks between 18-24 hours in aseptic wound models. Macrophages are then recruited to help in pathogen and debris clearance, as well as mediating transition to the *Proliferative phase*. This phase is marked by angiogenesis, re-epithelialization and collagen deposition. Lastly, wounds undergo a long *Remodeling phase*. Here, the initial collagen is crosslinked and remodeled in order to increase the tensile strength of the wound.

Antimicrobial Peptides as Early Mediators of Wound Healing

Integral to the host defense response at epithelial surfaces, AMPs serve to directly inhibit bacterial growth and help initiate recruitment of innate leukocytes (17, 133). As small amphipathic molecules, AMPs mediate bactericidal activity by integration and disruption of microbial membranes. Within the skin, several classes of AMPs, including cathelicidins (LL37/CRAMP) and β-defensins, have been identified to be important in response to both infection and injury (13, 134). Human β-defensin (hBD1) is constitutively expressed by keratinocytes, where as hBD-2/mBD-3 and hBD3/mBD14 are predominately upregulated following inflammation, tissue injury or infection (13-15). Namely, hBD2-4 have been shown to induce keratinocyte pro-inflammatory cytokine and chemokine secretion as well as enhancing keratinocyte migration and proliferation (135).

In settings of non-healing wounds, such as diabetic foot ulcers, expression of activated human cathelicidin, LL37, was reduced compared to tissue from healthy donor skin (136). In parallel to this, murine studies in $CRAMP^{-/-}$ mice report delayed wound healing. On the contrary, levels of hBD1-4 were elevated in tissue samples from non-healing and diabetic foot ulcers (136, 137). Interestingly, in a porcine model of *S. aureus* excisional wound infection, treatment with human hBD3 enhanced bacterial clearance and wound re-epithelialization as compared to controls (138). Together, these data suggest that alterations in AMP expression or function may contribute to aberrant healing in pathologic settings, and reconstitution of functional AMPs could provide therapeutic options for chronic, non-healing wounds.

Controversial Role of Neutrophils in Wound Repair

Neutrophils are typically described as the first class of infiltrating innate immune cells at the site of injury, helping to control potential infection by phagocytosis of foreign pathogens and removal of contaminating debris. Despite this necessary function, excessive release of enzymes by neutrophils, such as elastase, can be damaging to surrounding tissue, delaying rates of healing and increasing the risk of scar formation (65, 130, 139, 140). For these reasons, the need for neutrophil infiltration following injury is heavily debated. In support of neutrophil-dependent wound healing, mice lacking the CXCR2 receptor responsible for neutrophil chemotaxis have an expected impairment in neutrophil recruitment to the site of injury (141). Interestingly, this was accompanied by decreased re-epithelialization and angiogenesis (141). Despite insufficient neutrophil recruitment, monocyte and macrophage infiltration was actually enhanced in CXCR2 deficient mice (141), suggesting that loss of the neutrophil population may alter the important, yet ill-defined, cross-talk between these two cell subsets in the context of wound healing. Moreover, aging studies support the necessity for neutrophil infiltration for efficient wound closure. In these studies, neutrophil depletion further impairs rates of wound closure in aged mice (49). Additionally, topical application of isolated peritoneal neutrophils following injury or intravenous injection of G-CSF to mobilize neutrophils to the peripheral pool prior to wounding, enhanced rates of wound closure to that seen in young mice (49). These data imply that topical or systemic G-CSF treatment may provide a potential therapeutic avenue for neutropenic or immunocompromised individuals, such as cancer patients or the elderly, who may not be able to recruit adequate numbers of neutrophils to site of cutaneous injury and infection.

On the other hand, studies in young mice report a very divergent finding regarding neutrophil necessity in cutaneous repair. *In vivo* neutrophil depletion in young mice with rabbit anti-mouse sera prior to injury resulted in increased re-epithelialization out to day 3 post injury with no significant difference in macrophage infiltration (140). The evidence provided by Dovi *et al.* is supported by earlier findings that conclude neutrophils are either dispensable in cutaneous repair (142) or that their excessive presence is detrimental to the healing process (143). In the later study, impaired healing in a murine model of diabetes was associated with prolonged expression of MIP-2 and a concomitant extended infiltration of neutrophils at the site of injury (143). Also arguing against a positive effect of neutrophil recruitment to sites of aseptic injury are fetal wound healing models. Fetal wound healing is characterized by a pronounced decrease in inflammatory mediators and neutrophil recruitment at injury sites, minimal, if any scar formation, and swifter rates of wound closure (130, 131). While these data suggest that neutrophils may either be unnecessary or detrimental to prototypic wound repair, the aging studies provide an interesting point of contention. The differences observed in fetal, young and aged mice may actually highlight an aging difference reflective of the immunosenescent phenotype of the elderly, suggesting that modulation of wound closure by neutrophils may be required in the elderly yet detrimental *in utero* and in younger patients. While the current data are somewhat inconclusive regarding the positive and negative contribution of neutrophils to efficient wound repair, similar to many physiologic processes, it is highly likely that maintenance of a delicate balance is ideal for satisfactory wound repair.

Macrophages as Critical Mediators of Wound Healing

There is a general acceptance that macrophages are instrumental in prototypic wound healing, being involved predominately in the inflammatory and proliferative phases of wound repair. Their crucial role is highlighted as impaired macrophage function or alterations in macrophage infiltration kinetics and duration are associated with a prolonged inflammatory response, inadequate wound closure and poor restoration of normal cutaneous architecture (127, 144-148). The critical function of macrophages is emphasized by studies utilizing selective depletion of the macrophage population immediately prior to injury (144). In these studies, macrophage ablation was associated with increased TNF-α production and delayed re-epithelialization at 5 days post injury. These mice also exhibited decreased angiogenesis, as noted by diminished $CD31^+$ staining and reduced VEGF production, as well as impaired collagen production and decreased transforming growth factor –β (TGF-β) production, implying that macrophages help modulate the wound inflammatory milieu and progression into the proliferative phase of wound healing (144).

In addition to evidence supporting an absolute requirement for macrophages in efficient repair, reports suggest that macrophage phenotype plays a significant role in the modulation of the response to cutaneous injury (118, 145, 149). Following injury, peripheral blood monocytes extravasate to the wound site, undergoing differentiation into wound macrophages (79, 118). While initially thought of as alternatively activated, or M2-polarized, macrophages, emerging evidence supports that the first macrophages entering the wound site display a pro-inflammatory phenotype, marked by TNF-α and

IL-6 production (118). As the inflammatory phase progresses, wound macrophages increase production of TGF-β and express typical markers of alternative activation, including arginase1 and Ym1 (118). Interestingly, prolonged expression of classical markers of activation in wound macrophages is tied to failure of efficient wound resolution (145), implying that timely transition to an M2 phenotype is required for a competent physiologic response to cutaneous injury. Based on evidence that M2 macrophages enhance wound repair, recent studies have utilized mesenchymal stem cells (MSC) to drive M2 polarization (149). Zhang *et al*. found that co-culture of gingiva-derived MSCs could effectively home to sites of cutaneous injury, induce an M2 phenotype and remarkably accelerate wound closure (149). While studies exploring the therapeutic potential of stem cells in human and animal models of cutaneous injury have consistently provided evidence that MSCs promote wound repair (reviewed in (150)), this is one of the first reports to postulate a mechanism by which MSCs directly interact with wound immune cells to enhance closure.

Stem cell therapeutic interventions which augment macrophage function may be of particular interest in several patient populations, such as the elderly and diabetics (50, 64, 119, 145, 151, 152). These patients are often plagued by prolonged rates of wound closure that are attributable to impaired macrophage function (50, 143, 145, 147). Wound macrophages in diabetic models exhibit decreased phagocytic function and heightened pro-inflammatory cytokine production (147), which may impair resolution and elevate the risk of infectious complications in a clinical setting. This impaired phagocytic capability was specifically characterized as defective macrophage efferocytosis (147), or

phagocytosis of apoptotic cells (153, 154). Increased apoptotic cell content in wounds from diabetic mice was associated with an overall delay in wound closure. Culture of isolated wound macrophages from diabetic mice with apoptotic thymocytes demonstrated a reduced ability clear dead cells (147). In line with this study, diabetic murine models of excisional skin injury or skin flap cutaneous injury demonstrated increased levels of MCP-1 in cutaneous wounds (143, 145). Heightened production of this chemokine correlated with enhanced macrophage infiltration and delayed wound closure (143) or reintegration of the injured flap (145). These data parallel studies in aging mouse models, in which impaired cutaneous wound closure in aged mice is associated with elevated MCP-1, increased macrophage infiltration and the prolonged presence of macrophages at the site of injury (50). Taken together, these data highlight the absolute necessity for intact macrophage function to effectively modulate the inflammatory and temporal response to wound injury. Perhaps treatment of clinically compromised wounds with MSC could not only aid in transition to the anti-inflammatory phenotype seen in both diabetics and the elderly, but rescue defective efferocytosis and phagocytosis to further aid in wound repair. As the knowledge of macrophage involvement in wound repair advances and evolves, this particular innate immune cell subset is likely to emerge as a key candidate for targeted intervention and treatment in non-healing, chronic wounds in at risk patient populations.

Langerhan's and Mast Cells in Cutaneous Wound Healing

Two additional innate leukocytes have been shown to have a hand in modulation of wound resolution, Langerhan's cells (LC) and mast cells (MC). LCs, resident dendritic

cells of the epidermis, have been shown to have differential responses to two types of non-healing cutaneous ulcers: venous leg ulcers and diabetic foot ulcers (137). In patients with venous leg ulcers there was a decreased accumulation of $CD1a^+$ LC at the site of injury as compared to aged matched controls. Conversusly, there was a marked increase in $CD1a^+$ LC in patients with diabetic foot ulcers in comparison to controls. The authors attributed this difference to increased expression GM-CSF in keratinocytes present in diabetic foot ulcers (137). In an alternate study, enhanced LC accumulation was actually tied to excessive scar formation, as opposed to chronic wounds (155). Niessen *et al.* found that the number of LC in the epidermis was elevated in hypertrophic scars at 3 months after surgery, though no difference was noted at 12 months (155). Clearly these studies outline that LC aid in regulation of wound repair, but their precise functional contributions are yet to be delineated.

Mast cells, another epidermal resident leukocyte with known functions in autoimmune and inflammatory pathology, have been postulated to play a role in cutaneous wound healing (156-158). Studies utilizing MC-deficient mice have found that absence of MC delays wound closure and results in dysregulation of the inflammatory stage of wound healing through impaired neutrophil recruitment (157, 158). Weller *et al*. extended these findings by examining the role of MC histamine release as a potential mediator of the impaired wound closure seen in MC-deficient mice. These studies found that blockade of histamine 1 receptors impaired wound closure, and suggested that histamine release by activated MC may mediate the positive effect of MC on cutaneous healing (157). As histamine, a potent vasodilator, is common place in clinical practice,

topical application may serve as yet another targeted therapeutic to enhance wound closure and improve clinical outcomes following tissue injury.

Summary of Innate Immune Cells as Mediators of Wound Healing

The physiologic response to cutaneous injury is undoubtedly a complex, highly regulated process. Likewise, the contribution of innate immune leukocytes and lymphocytes to this efficient wound repair process is multifaceted. As these cells are some of the earliest responders and mediators of the local response to cutaneous injury, they serve as ideal targets for modulation of the wound repair process. Early intervention in at risk patient populations with novel therapeutic strategies derived from our understanding of the innate mediators of tissue injury can potentially decrease the incidence and prevalence of chronic, non-healing wounds, reduce infectious complications and ameliorate associated health care costs.

Overall Significance:

Age-Associated Alterations in Innate Immune Function and Wound Repair

Increased human life expectancy over the last century has resulted in growth of the aging population; however, the prevalence of disease and associated morbidities among the elderly has also increased (1). Older patients are more susceptible to post-surgical infections and subsequent problematic wound closure with costly prolonged hospital stays (121, 159). In addition, these patients suffer from chronic wound states that offer persistent sites for infection by foreign pathogens (119, 151), potentially propagating infectious spread in long-term care settings. By determining how aging impairs innate immune cell function, novel therapeutic approaches that aid in resolution of wound infection may be realized, resulting in improved post-surgical outcomes, diminished rates of chronic wounds and a clinically significant reduction in morbidity and mortality in the elderly.

CHAPTER 3

REDUCED NEUTROPHIL CHEMOTAXIS AND INFILTRATION CONTRIBUTES TO DELAYED RESOLUTION OF CUTANEOUS WOUND INFECTION WITH ADVANCED AGED

Abstract

Advanced age is associated with alterations in innate and adaptive immune responses, which contribute to an increased risk of infection in elderly patients. Coupled with this immune dysfunction, elderly patients have elevated rates of wound dehiscence, chronic wounds and wound infection, suggesting that aging impairs wound healing. To evaluate how advanced age alters the host immune response to cutaneous wound infection, we developed a murine model of cutaneous *S. aureus* wound infection in young (3-4 month) and aged (18-20 month) BALB/c mice. Aged mice exhibit increased bacterial colonization and delayed wound closure over time compared to young mice. These differences were not attributed to alterations in wound neutrophil or macrophage TLR2 or FcγRIII expression, or age-related changes in phagocytic potential and bactericidal activity. However, reduced neutrophil and macrophage recruitment was observed in aged mice relative to young mice despite elevated KC, MIP-2 and MCP-1 chemokine levels at the wound site. In response to a subcutaneous injection of KC, aged mice recruited fewer neutrophils at increasing doses of KC compared to young mice, suggesting age impairs *in vivo* neutrophil chemotaxis to cutaneous tissue. These age-mediated defects in early

neutrophil recruitment may alter the dynamics of the inflammatory phase of wound healing, impacting macrophage recruitment, bacterial clearance and wound closure.

Introduction

An estimated 25 billion dollars in US health care expenditure is spent on care of chronic, non-healing wounds (151). One patient sub-population, the elderly, account for a major portion of patients afflicted by chronic and infected wounds (119, 151, 159). Aging is associated with a decline in immune function which can elevate rates of wound infection and delay wound closure in these patients (64, 160, 161). Moreover, advanced age is associated with a general decline in innate and adaptive immune function which contributes to an increased susceptibility to opportunistic bacterial and viral infections in the elderly (6, 159, 162, 163). Underlying this elevated susceptibility to infection are deficits in host recognition, phagocytosis, migration and activation of a pathogen-specific adaptive immune response (3, 45, 83, 164). In addition to the immunosenescence that accompanies aging, advanced age is also marked by heightened levels of circulating pro-inflammatory mediators in the absence of an inciting stimulus, a state referred to as "inflamm-aging (56, 165)." Together, these phenomena are thought to contribute to poor outcomes following infection or trauma in clinical and animal models of aging (47, 48, 89, 159, 166, 167).

Alongside playing a critical role in eradication of foreign pathogens, the innate immune response is also crucial to tissue repair, another physiologic response compromised by aging. Cutaneous wound repair is a complex process, marked by immune cell infiltration and inflammation, fibroblast and keratinocyte proliferation, angiogenesis, and remodeling of the extracellular matrix to ensure restoration of the

normal skin barrier (118, 139, 160, 168, 169). Natural and succinct progression through these interdependent stages of wound healing is required to ensure rapid and adequate repair of epidermal and dermal architecture. Clinical observations reveal that elderly patients have an impaired response to cutaneous injury, resulting in elevated rates of wound dehiscence and subsequent chronic wounds (121, 151). Non-healing wounds in this patient population have an increased risk of infectious complications. This risk is associated with higher morbidity and mortality, and infectious spread in long-term care facilities (120). Moreover, older patients are more susceptible to post-surgical infections and subsequent problematic wound closure with costly prolonged hospital stays (121, 159).

Laboratory studies using murine models of cutaneous injury have substantiated the aforementioned clinical findings as advanced age has been shown to alter the dynamics of all stages of the wound repair process (49, 50, 63, 170). Evidence suggests age-associated differences in the magnitude and duration of neutrophil and macrophage infiltration into the wound bed during the inflammatory phase of wound healing (49, 50). These phagocytes are instrumental in limiting infectious spread and regulation of the temporal progression through the early phases of wound closure (118, 171, 172). Despite compromised immune function and increased rates of infectious wound complications in the elderly, the impact of advanced age on the host immune response to cutaneous wound infection has been neglected. *S. aureus* is a common dermatopathogen that accounts for at least 75% of skin and soft tissue infections and 30% of surgical-site infections, with a dramatic increase in *S. aureus*-associated surgical site infections to 50% in patients over 65 (159, 173, 174). We developed a cutaneous excisional *S. aureus* wound infection

model in young (3-4 month) and aged (18-20 month) BALB/c mice. In the setting of wound infection, we are the first to demonstrate that advanced age is associated with heightened bacterial colonization and delayed wound closure due to reduced innate immune cell recruitment.

Materials and Methods

Animal Model

3-4 month old (young) and 18-20 month old (aged) BALB/c mice (Charles River/NIA, Kingston Facility, Stony Ridge, NY) were utilized to determine age-dependent differences in response to cutaneous wound injury and infection (**Figure 5**). All animal studies were approved and performed with strict accordance to the regulations established by the Loyola University Chicago Animal Care and Use Committee. Following acclimation at Loyola's Animal Care Facility, young and aged mice were subjected to dorsal excisional cutaneous injury as previously described (175). Briefly, mice were administered intraperitoneal (*i.p.*) 100 mg/kg ketamine and 10 mg/kg xylazine followed by *i.p*. saline to ensure systemic distribution of the anesthetic. Once the mice no longer responded to firm pressure applied to their hind limb, their dorsum's were shaved and cleansed with ethanol pads. Mice were then subjected to 6 dorsal full-thickness (skin and panniculus carnosa) cutaneous wounds with a 3 mm dermal punch biopsy (Acuderm, Ft. Lauderdale, FL). Immediately after injury, mice received ~10^3 colony forming units (CFU)/10uL of *S. aureus* to each wound and were returned to their cages on heating pads. A low inoculum of bacteria was chosen to prevent sepsis which is known to negatively impact wound healing (176). *S. aureus* Newman strain was grown overnight in tryptic

soy broth (TSB) at 37^{o}C under constant agitation. The next day, 1 mL of *S. aureus* in TSB was resuspended in 2mL fresh TSB and incubated at 37^{o}C for 2 hours to ensure mid-logarithmic growth at the time of application to cutaneous wounds. Bacterial concentration (CFU/mL) was determined by absorbance at 600 nm and the final inoculum confirmed by back-plating on mannitol salt agar (MSA; BD Diagnostics, Sparks, MD).

Mice were sacrificed at days 1, 3, 7 and 10 after injury and infection. The pelt was removed and photographed to measure wound size as described below. A larger 5 mm punch biopsy was used to remove the 3 mm wounds. Wounds were examined for bacterial colonization, *ex vivo* phagocytosis, flow cytometric analysis of wound immune infiltrate and cytokine and chemokine analysis as detailed below. Blood was obtained via cardiac puncture for flow cytometric analysis, as well as the bactericidal assay.

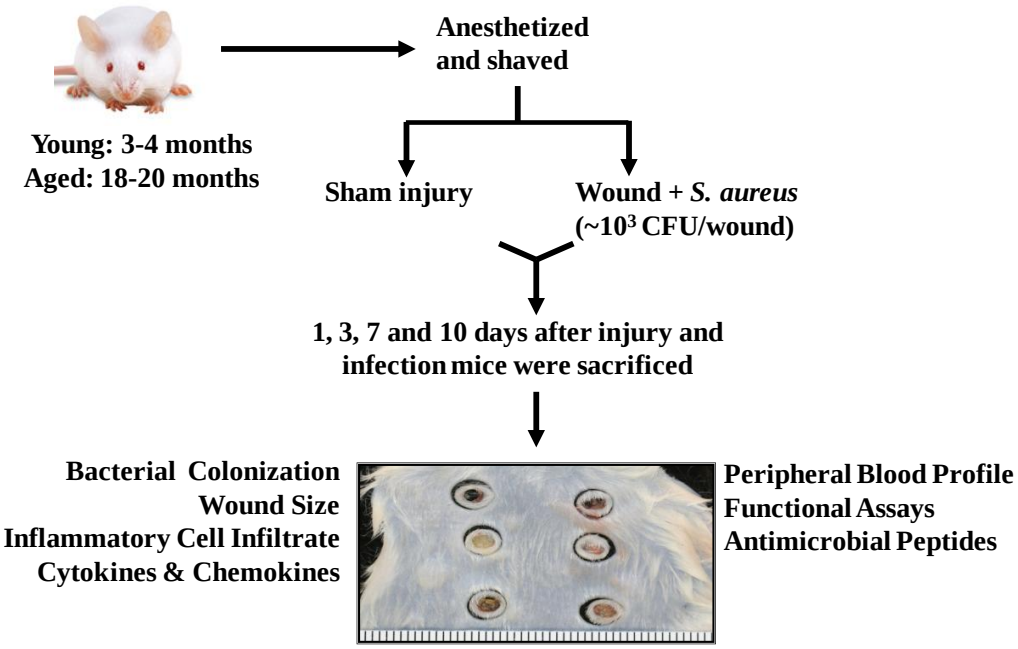


Figure 5. Model of *S. aureus* cutaneous wound infection in young and aged mice.

Young (3-4 month) and aged (18-20 month) BALB/c mice were anesthetized and their dorsum shaved. Mice then received 6, 3 mm dorsal punch wound followed by ~10^3 CFU of *S. aureus* per wound. Mice were sacrificed at days 1, 3, 7 and 10. Wounds were assessed for bacterial colonization, wound size, inflammatory cell infiltrate, cytokines, chemokines, phagocytosis and AMPs. The peripheral blood was harvested for evaluation by flow cytometry.

Bacterial Colonization

Skin, spleen and kidney were each homogenized in 1 mL sterile phosphate buffered saline (PBS) and 10-fold serial dilutions to 10^6 were plated on MSA plates (BD Diagnostics, Sparks, MD). Heparinized whole blood was directly plated onto MSA plates. Plates were incubated at 37^{o}C for 24-48 hours and colonies were counted to determine levels of bacterial colonization. Significant bacterial dissemination to spleen, kidney and blood was not observed (data not shown).

Wound Size

Wound size was evaluated by digital photography and image analysis as previously described (175). Briefly, at days 1, 3, 7 and 10 wounds were photographed with a Canon EOS SLR digital camera. Each pelt photographed at a fixed distance of 20 cm with a ruler placed within the frame of each photograph. Photoshop 7.0 (Adobe Systems Inc., San Jose, CA) was used to determine the number of pixels in the open wound area using the magic wand tool, with zoom at 100% and a tolerance setting of 60. Separate animals were sacrificed immediately following wound injury and wound size was determined to represent day 0. Wound areas at each time point were compared with day 0 wounds: (pixels at days1-10/pixels at day 0)^{x}100 were used to determine the percent open wound area at each time point.

Skin and Whole Blood Flow Cytometry

Single cell suspensions of wound cells for flow cytometry were generated as previously described (177). At days 1-7 after injury and infection, animals were

euthanized, pelts removed and wounds excised using a 5 mm punch biopsy. Diced wounds were incubated overnight at 4^{o}C in RPMI 1640 culture media containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM L-glutamine (Gibco, Grand Island, NY), 1% penicillin/streptomycin (Gibco, Grand Island, NY), 2 mg gentamycin sulfate (Mediatech Inc, Manassas, VA) and 0.3 mg dispase II (Roche Diagnostics, Indianapolis, IN). The next day, tissue pieces were removed and subjected to further enzymatic digestion with 1 mg collagenase from *Clostridium histolyticum* type 1A (Sigma-Aldrich, St. Louis, MO), 1.2 mg DNAse I from bovine pancreas Grade II (Roche Diagnostics, Indianapolis, IN), 1 mg hyaluronidase from bovine testes type 1-S (Sigma-Aldrich, St. Louis, MO), in RPMI 1640 with 5% FBS, 2 mM L-glutamine (Gibco, Grand Island, NY), 1% penicillin/streptomycin (Gibco, Grand Island, NY), 2 mg gentamycin sulfate (Mediatech Inc, Manassas, VA) and magnesium chloride hexahydtrate for 2 hours at 37^{o}C. After two hours, these solutions were combined and debris removed by filtration with a 70 μm filter. Cells that remained adherent to the tissue culture plastic were treated with Accutase (eBioscience, San Diego, CA) for 5-8 minutes at 37^{o}C followed by vigorous pipetting. Cells were washed and adjusted to 1x10^{6}/mL. Cells were blocked for 20 minutes with FcBlock (anti-CD16/CD32, eBioscience) and rat IgG (Jackson Immuno), and then stained with PE-Cy7-F4/80 (eBioscience), FITC-Gr-1 (eBioscience, San Diego, CA), APC-CD3 (eBioscience) or APC-TLR2 (eBioscience, San Diego, CA). Cells were washed twice and then resuspended in flow buffer (1% bovine serum albumin (BSA), 0.1% sodium azide and 2mM EDTA in PBS). For blood analysis, heparinzed whole blood was collected and stained per the manufacture's protocol (Leinco Technologies). Whole blood (100 μL) was blocked for 20 minutes with FcBlock and rat IgG and then

stained with PE-Cy7-F4/80, FITC-Gr-1 and PE-CXCR2. Following staining, erythrocytes were lysed with 2 mL Easy Lyse Solution (Leinco Technologies) for 11.5 minutes. Lysis was terminated by addition of 2 mL ice cold Wash Buffer (Leinco Technologies). Cells were washed twice in flow buffer and resuspended in 500 μL flow buffer. Both skin and blood samples were collected on the FACSCanto I and FACS LSR Fortessa (BD Bioscience, San Jose, CA) and data were analyzed by FlowJo Software (Tree Star Inc, Ashland, OR). Fluorescence minus one control staining and single color controls were used to determine positive staining.

Phagocytosis and FcγRIII Staining

Following isolation, wound cells were subjected to pHrodo-*S. aureus* phagocytosis as previously described (177). Cells were resuspended to $1x10^6$ cells/mL in Phagocytosis Uptake Buffer (Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY), with 20 mM HEPES, pH 7.4) per the manufacturer's instructions (Invitrogen, Carlsbad, CA). pHrodo-*S. aureus* BioParticles (Invitrogen, Carlsbad, CA) were reconstituted to 1 mg/mL in Phagocytosis Uptake Buffer and then opsonized with rabbit polyclonal IgG antibodies (Invitrogen, Carlsbad, CA) for 1 hour at 37^{o}C. Control tubes for each animal were placed on ice (4^{o}C) and experimental tubes placed at 37^{o}C for 15 minutes to allow for temperature equilibration. pHrodo-*S. aureus* was then added at a ratio of 30:1 bacteria particles to cell. Samples were incubated for 0-60 minutes at 4^{o}C (control) or 37^{o}C (experimental) after which phagocytosis was stopped by addition of 2 mL ice cold Phagocytosis Uptake Buffer and placement on ice. Cells were blocked with rat IgG and anti-FcγRII (R&D Systems, Minneapolis, MN), to block FcγRI and FcγRII, respectively,

for 20 minutes at 4^{o}C. Samples were then stained with PE-anti-FcγRIII (R&D Systems, Minneapolis, MN) for 30 minutes followed PE-Cy7-anti-F4/80 and FITC-anti-Gr1 for 30 minutes at 4^{o}C (178). Cells were washed twice, resuspended in flow buffer, and phagocytosis and FcγRIII expression on wound neutrophils and macrophages were examined by flow cytometry. Data were acquired on the FACSCanto1 (BD Bioscience, San Jose, CA) and analyzed by FlowJo Software (Tree Star Inc, Ashland, OR).

Peripheral Blood Neutrophil Isolation and Bactericidal Assay

Heparinized whole blood was collected from unmanipulated animals and neutrophils sorted on the FACSAria. Briefly, 500-700uL heparinized blood was lysed according to the manufacturer's instructions (Leinco Technologies). Cells were washed twice and resuspended in flow buffer. Cells were sorted based on forward (FSC) and side (SSC) scatter. Cytospins and Diff Quick staining of sorted cells were used to confirm purity by morphology. Sorted cells were evaluated for bactericidal activity as previously described with some modifications (179). Neutrophils were seeded at 10^5 cells onto poly-L-lysine coverslips for 1 hour at 37^{o}C. *S. aureus* Newman was grown overnight in TSB, resuspended to 10^6 CFU/mL in TSB and opsonized with 5% normal mouse serum in HBSS for 30 minutes at 37^{o}C. Coverslips with incubated with bacteria (10:1 ratio of bacteria: cells) in HBSS for 15 minutes under agitation to allow phagocytosis to occur. Phagocytosis was stopped by placing coverslips in ice cold HBSS and washing three times. One coverslip was then lysed with ice cold ddH_20 for 5 minutes. Lysates and 10-fold serial dilutions of lysates were plated on MSA plated for 24-48 hours to determine the amount of bacteria phagocytosed. The remaining coverslips were placed in HBSS for

45 or 75 minutes at 37^{o}C to allow bactericidal activity to occur. At either 45 or 75 minutes, coverslips were lysed with ice cold ddH_20. Lysates and 10-fold serial dilutions of lysates were plated on MSA and incubated at 37^{o}C for 24-48 hours. Bacterial growth was determined by colony counts. Bactericidal activity (percent killing) was determined by: (number of colonies remaining at either 45 or 75 minutes/number of colonies initially present after the phagocytosis phase)*100.

Chemokine Analysis

One wound was homogenized in 1mL BioRad Cell Lysis buffer (BioRad, Hercules, CA) supplemented with Factor 1, Factor 2 and PMSF per the manufacturer's instructions (BioRad, Hercules, CA). Homogenates were sonicated at 30%, syringe-filtered (25 □m) and chemokines (KC, MIP-2 and MCP-1) were analyzed by ELISA (R&D Systems, Minneapolis, MN) (180).

In Vivo Chemotaxis

Young and aged mice were administered 100 mg/kg ketamine and 10 mg/kg xylazine followed by saline to ensure systemic distribution of the anesthetic. Once the mice no longer responded to firm pressure applied to their hind limb, their dorsum's were shaved and cleansed with ethanol pads. In the dorsal subscapular midline, mice received subcutaneous (*s.c.*) injections of 100 or 1000 pg of recombinant mouse KC in a volume of 50 μL or saline vehicle (50 μL). Mice were sacrificed 8 hours after injections. The injection site and surrounding tissue were excised with an 8 mm punch biopsy and subjected to digestion as outlined above (177). Neutrophil recruitment to the injection site

was evaluated by flow cytometry as described above. Data are expressed as fold change of baseline neutrophil recruitment after saline injections.

Statistical analysis

Data are shown as mean ± standard error of the mean (SEM) of each group. Data were analyzed by Student's *t* test or one- way or two-way ANOVA with Tukey's or Bonferroni post-hoc tests, respectively, where appropriate using GraphPad Prism 5 (GraphPad, La Jolla, CA). A value of $p \leq 0.05$ was considered significant.

Results

Bacterial colonization and wound closure is impaired with advanced age.

To evaluate the impact of advanced age on the innate immune response following cutaneous injury and *S. aureus* infection, we developed a model of cutaneous wound infection in young and aged mice to evaluate bacterial colonization and wound size at days 1, 3, 7 and 10 after injury and infection (**Figure 5**). Bacterial colonization in wounds from aged mice were elevated over the time course examined as compared to young mice, with post-hoc significance observed at day 3 (**Figure 6**, $p<0.05$). Levels of wound bacterial burden were unaltered in aged animals at day 7 as compared to day 3, whereas gradual reduction in wound bacterial content was observed in young animals by day 7. Moreover, by day 10, wounds from young mice averaged 10^3 CFU/mL, compared to 10^6 CFU/mL in aged animals.

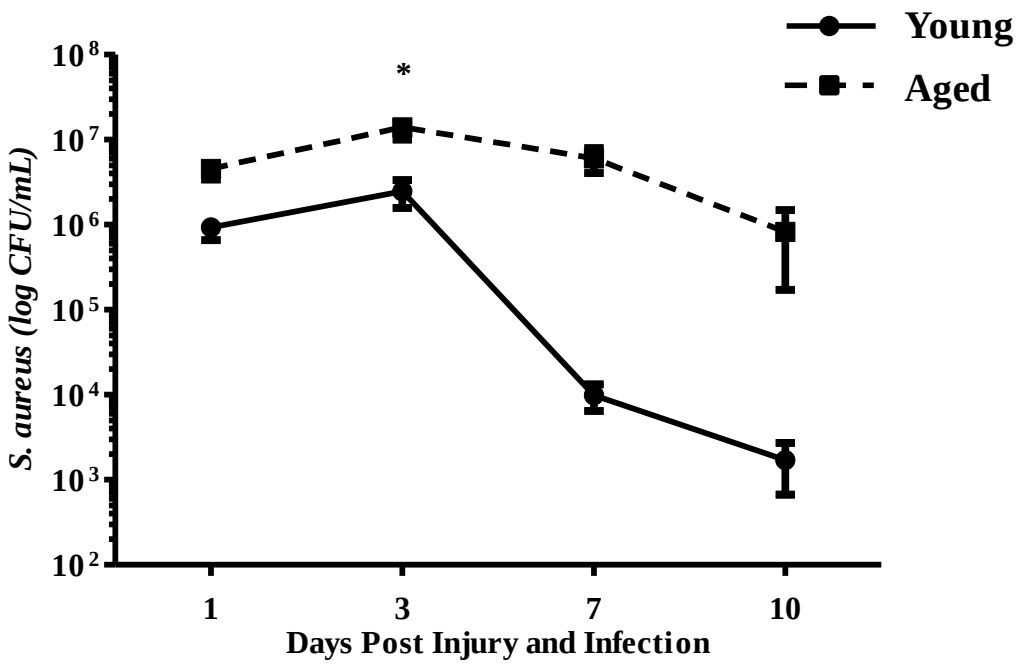


Figure 6. Wound bacterial colonization at days 1 through 10 following cutaneous injury and *S. aureus* infection. Young BALB/c (3-4 months, black circles and solid line) and aged Balb/C (18-20 months, black squares and dotted line) received six, 3mm dorsal cutaneous wounds followed by ~10^3 CFU *S. aureus*/wound. At days 1, 3, 7 and 10 after injury and infection, mice were sacrificed and bacterial colonization was determined by growth on MSA plates. Data are shown on a log scale as mean ± SEM, *$p<0.001$ compared to young at same time point by two-way ANOVA; N=8-20 per group at each time point.

In parallel to these studies, wound size was evaluated by digital photography and image analysis. Compared to young mice, wounds from aged mice had a larger percent open wound area remaining at days 3 and 7 following injury and infection (**Figure 7**, $p<0.05$), with incomplete closure at day 10. At day 3, wounds in aged animals remained ~80% open as compared to ~66% in young mice (**Figure 7B**, $p<0.05$). By day, an ~ 2-fold increase in percent open wound area was observed in aged mice as compared to young (**Figure 7B**, $p<0.05$). The persistent open wound bed at day 10 in aged animals (~11% versus ~0.9% in young mice) could result in subsequent infectious complications in aged mice. Together, these findings recapitulate clinical observations of increased infectious complications and delayed healing with advanced age.

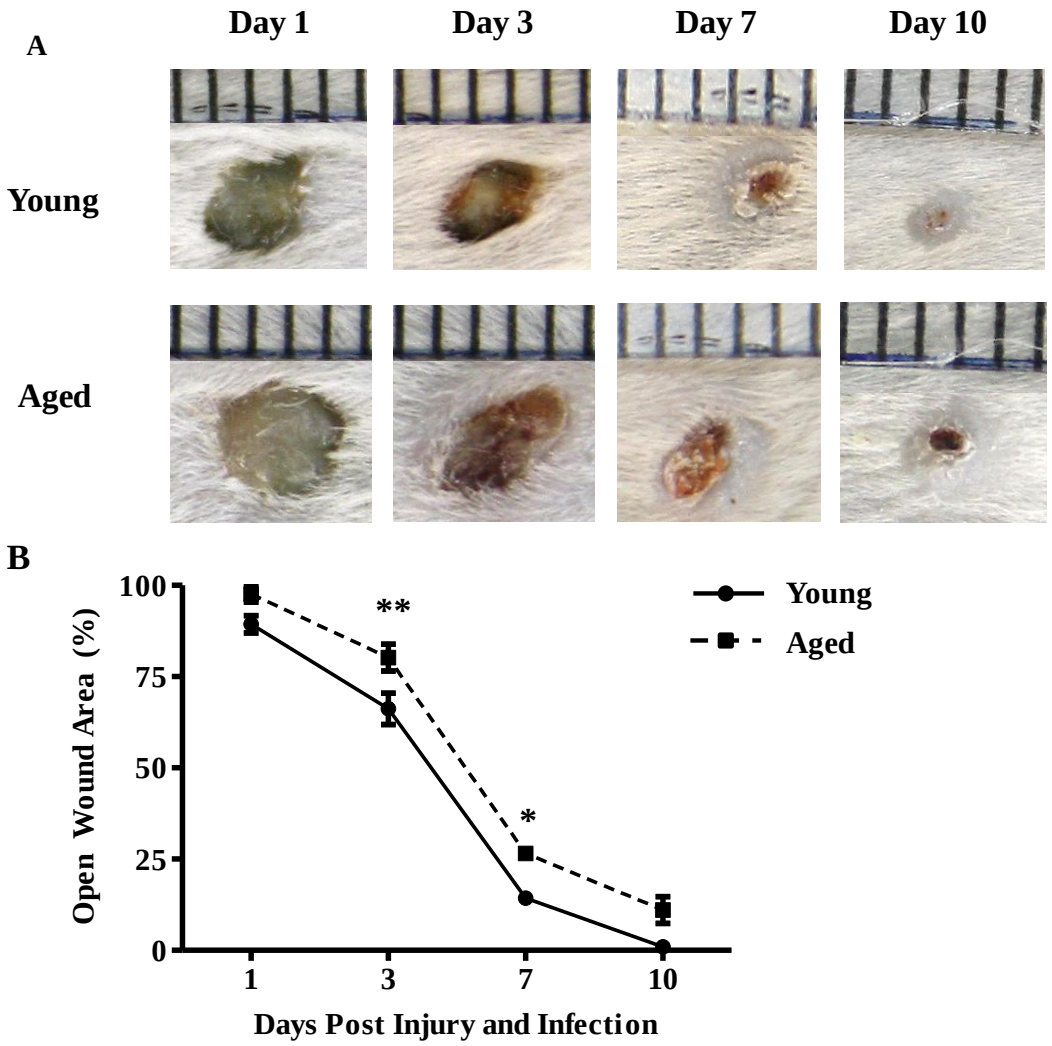


Figure 7. Wound size at days 1 through 10 following cutaneous wound infection with *S. aureus*. (**A**) Representative images of wound from young (top panel) and aged (bottom panel) mice are days 1, 3, 7, and 10 after injury and infection. (**B**) Wound size expressed as percent open wound area relative to time zero at days 1, 3, 7 and 10 after cutaneous wound infection in young (black circle and solid line) and aged (black squares and dotted line) mice. Data are shown as mean ± SEM, **$p<0.01$ and *$p<0.05$ compared to young at same time point by two-way ANOVA; N=7-15 per group.

Host recognition is not altered following injury and infection

Previous studies have reported decreased TLR expression in various innate immune cell subsets with advanced age (reviewed in (3, 161)). TLR-1, -2 and -6 play critical roles in early host recognition of Gram-positive pathogens, and loss of TLR2 or downstream mediators of TLR signaling, such as MyD88, results in increased susceptibility to *S. aureus* infection (98, 181-183). Given differences in bacterial colonization at day 3, we chose to evaluate TLR2 expression on resident tissue macrophages ($F4/80^{+}Gr\text{-}1^{-}$ cells from uninjured skin) as well as on infiltrating wound leukocytes in aged mice relative to young (**Figure 8**). In aged mice, a reduction in the absolute number of $TLR2^{+}$ resident tissue macrophages (**Figure 8B**, $p<0.05$) was observed compared to young mice. Following injury and infection, there were no age-dependent changes in the frequency of $TLR2^{+}$ infiltrating macrophages and neutrophils (**Figure 8C-D**). Moreover, aging did not alter the expression of TLR2 on infiltrating leukocytes after injury and infection (**Table 1**). The changes in number of $TLR2^{+}$ resident tissue macrophages with age may increase susceptibility to infection; however, following injury and infection, TLR2 expression did not contribute to prolonged bacterial infection in aged animals.

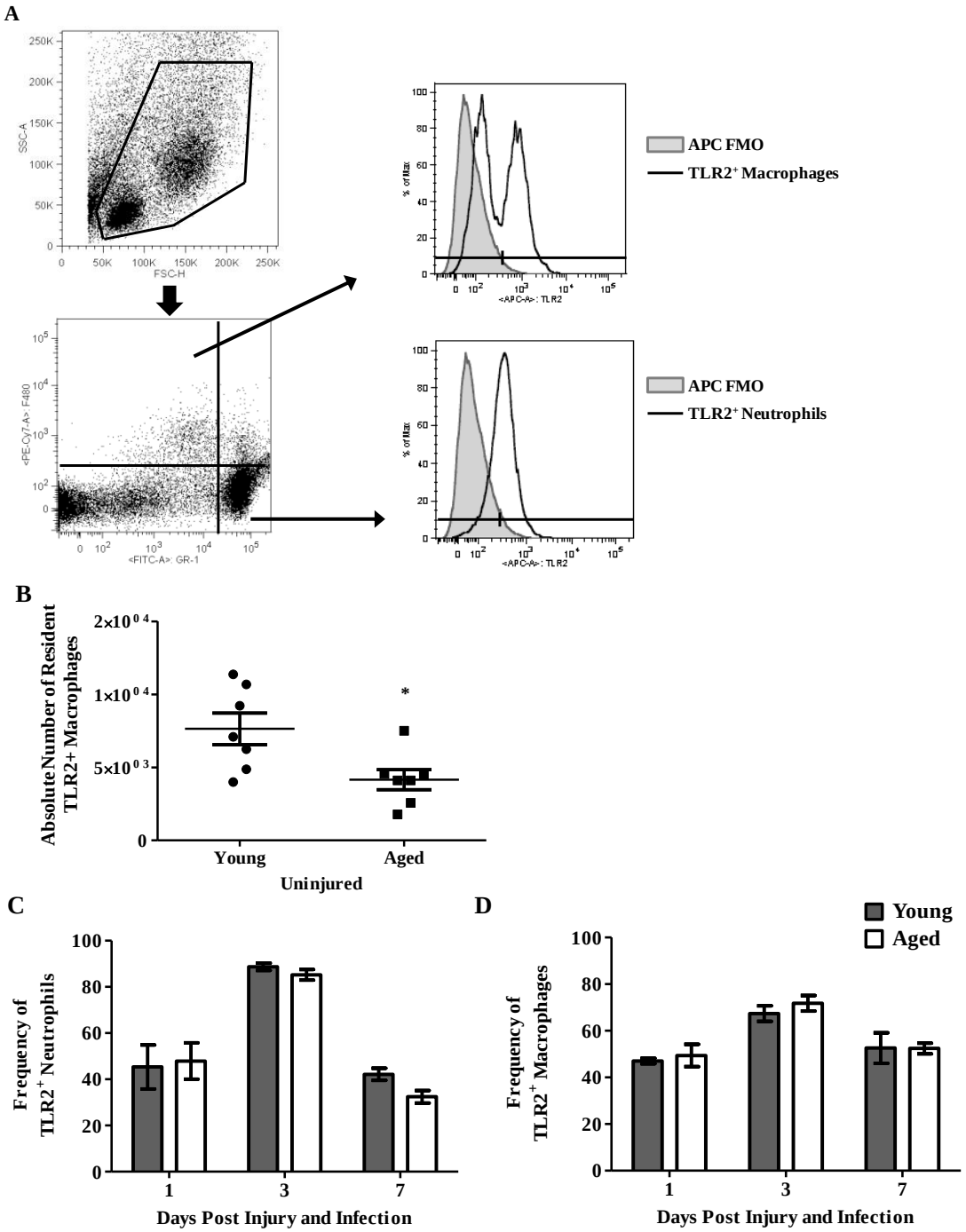


Figure 8. Expression of TLR2 by cutaneous neutrophils and macrophages. The number of neutrophils or macrophages expressing TLR2 was examined by flow cytometry. (**A**) Gating strategy for $TLR2^+$ resident and infiltrating leukocytes. Live cells

were gated on Gr-1 and F4/80; F4/80$^-$Gr-1$^+$ (neutrophils), F4/80$^+$Gr-1$^-$ (macrophages). Neutrophil and macrophage populations were then evaluated for TLR2 positivity (dotted black line). (**B**) Absolute number of TLR2$^+$ macrophages in uninjured skin from young and aged mice, *p<0.02 by Student's t-test, N=7 per group. (**C**) Frequency of TLR2$^+$ wound macrophages at day 1 and day 3 in young (gray bars) and aged (white bars) mice. (**D**) Frequency of TLR2$^+$ neutrophils at day 1 and day 3 in young and aged mice. Data are shown as mean ± SEM; N=8-17 per group. Data are not significant.

Table I. MFI of TLR2 by cutaneous neutrophils and macrophages.

	Macrophages (F4/80$^+$/Gr-1$^-$/TLR2$^+$)			**Neutrophils (F4/80$^-$/Gr-1$^+$TLR2$^+$)**	
	Uninjured	**Day 1**	**Day 3**	**Day 1**	**Day 3**
Young	518.7±22.2	678.3±48.1	603.0±17.0	612.1±39.1	451.6±15.9
Aged	586.3± 42.0	627.5±42.3	642.2±41.5	576.0±30.5	441.6±13.2

No difference was observed in the MFI of TLR2 in macrophages isolated from uninjured skin, or in the MFI of TLR2 in neutrophils or macrophages isolated from infected wounds. No significant differences between young and aged at each time point were observed. Data are shown as mean ± SEM; N=7-11 per group.

Bacterial clearance is not affected by advanced age

In addition to recognition of bacteria by the host immune system, neutrophils and macrophages that are recruited to the wound site are required to phagocytose and clear invading organisms. In particular, deficiencies in neutrophil and macrophage phagocytosis, as well as bactericidal potential, have been associated with reduced bacterial clearance as well as chronic or repeated infections (43, 46, 120, 184). A major phagocytic pathway in these cells is the FcγR pathway (32). Interestingly, reduced expression of FcγRIII (CD16) and phagocytosis have been observed in neutrophils from aged humans (46). Similar studies in macrophage populations suggest a reduced phagocytic potential with advanced age (185-187). Thus, we sought to determine if differences in phagocytosis and FcγRIII expression may contribute to delayed resolution of bacterial clearance in our model. Initially, we examined FcγRIII expression in peripheral blood neutrophils in our young and aged animals pre- and post-infection. In line with studies by Butcher *et al.*, there was a reduction in the frequency of FcγRIII^{+} neutrophils in aged mice following injury and infection (**Figure 9**)(46).

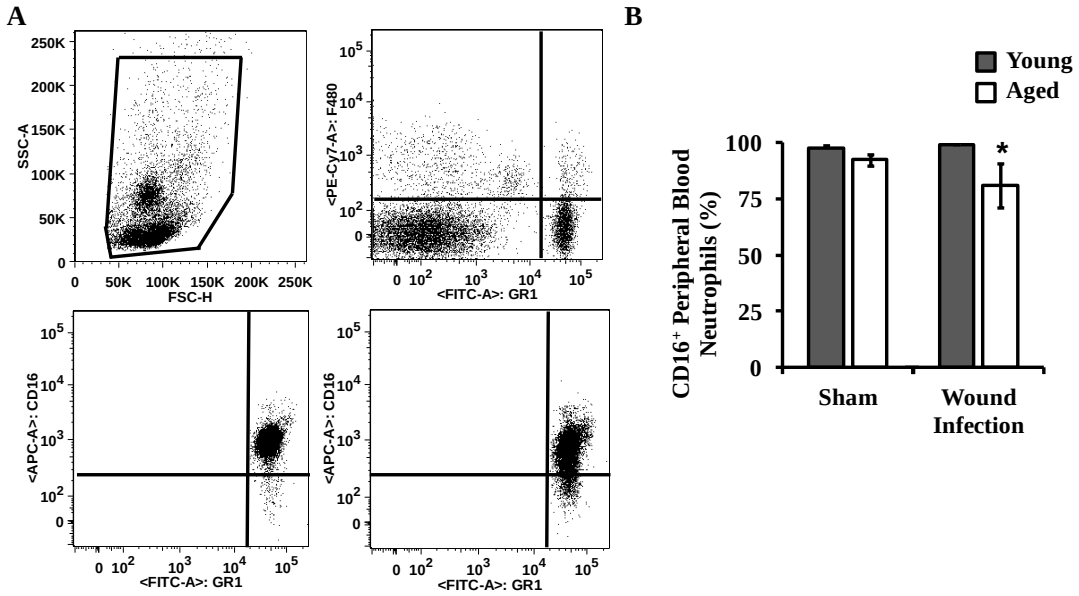


Figure 9. Expression of FcγRIII by peripheral blood neutrophils after injury and infection. Peripheral blood cells were stained for FcγRIII expression by flow cytometry. (**A**) Gating of FcγRIII^{+} neutrophil population (F4/80^{-}Gr-1^{+}FcγRIII^{+}). (**B**) Percentage of peripheral blood neutrophils expressing FcγRIII in young (gray bars) and aged (white bars) mice. Data are shown as mean ± SEM, *$p<0.05$ by one-way ANOVA with Tukey's t-test; N=3-4 per group.

Butcher *et al*. had documented that reduced FcγRIII expression was associated with reduce phagocytic potential in neutrophils isolated from the periphery of elderly subjects (46). Interestingly, few studies have examined the impact of the wound microenvironment on phagocytic cell function in the setting of advanced age. Given these data, we examined the phagocytic potential and the expression of FcγRIII pre- and post-phagocytosis in wound cells isolated from young and aged mice. Phagocytosis of pHrodo-*S. aureus* bioparticles by wound neutrophils were not different in young and aged mice (**Figure 10A-B**). Despite no observed changes in phagocytosis, there were fewer FcγRIII^{+} neutrophils pre- and post- phagocytosis in aged animals and the MFI of FcγRIII was diminished (**Figure 10C-D**, $p<0.05$).

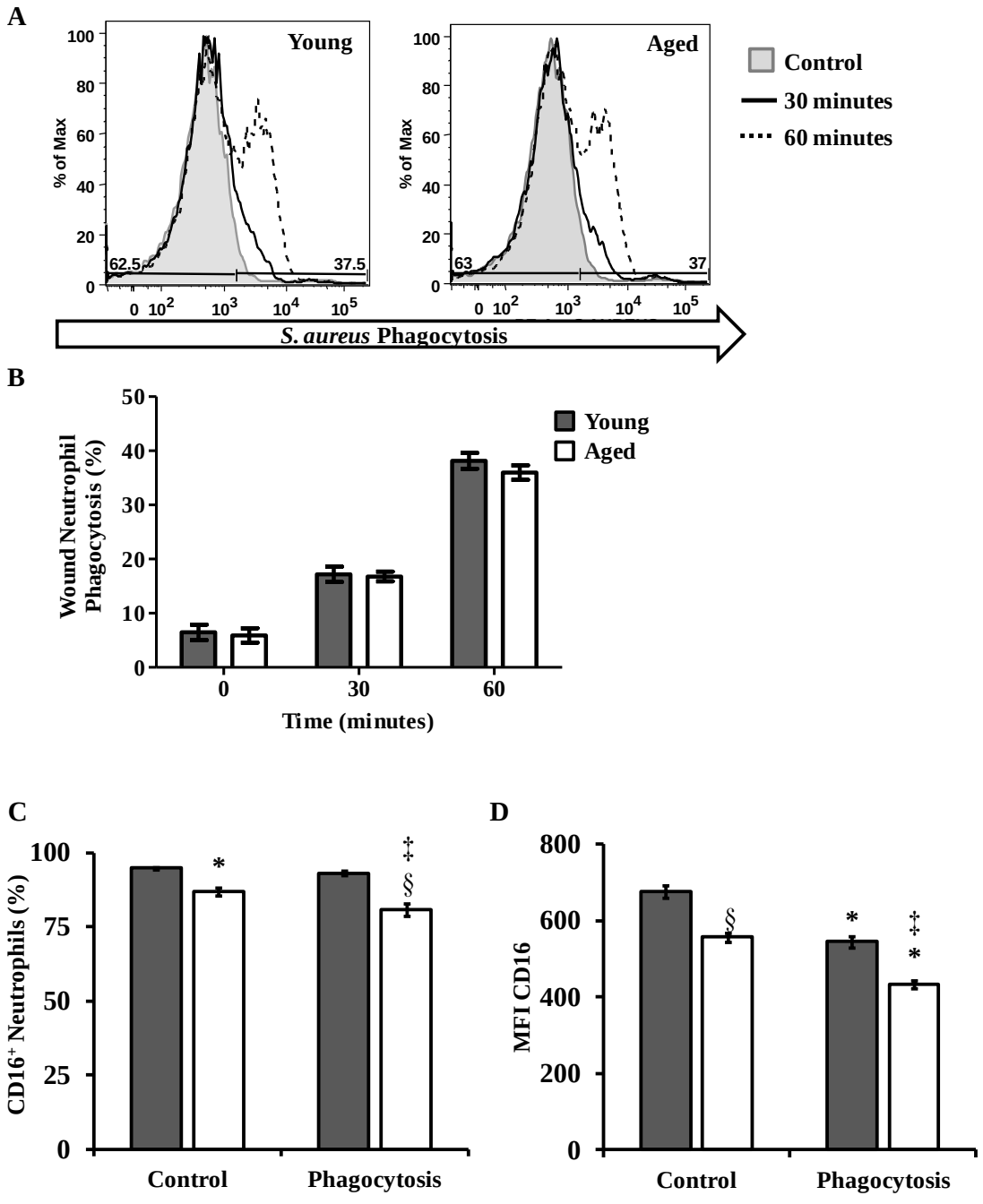


Figure 10. Wound neutrophil phagocytosis of *S. aureus* and FcγRIII expression.

Wound leukocyte suspensions were allowed to phagocytose pHrodo-*S. aureus* particles for 0-60 minutes and then stained for FcγRIII expression. Phagocytosis and FcγRIII were

assayed by flow cytometry. (**A**) Representative histograms (shaded gray-4^{o}C, control; black-37^{o}C, 30 minutes; and black dotted-37^{o}C, 60 minutes) of phagocytosis by wound neutrophils (F4/80^{-}Gr-1^{+}*S. aureus*$^{+}$). (**B**) Percentage of wound neutrophils that phagocytosed pHrodo-*S. aureus* particles in young (gray bars) and aged (white bars) mice. Data are not significantly different. (**C**) Percentage of wound neutrophils expressing FcγRIII in young and aged mice; *$p<0.05$ versus young control, ‡$p<0.001$ versus young phagocytosis, §$p<0.05$ versus young and aged controls by one-way ANOVA. (**D**) MFI of FcγRIII on wound neutrophils from young and aged mice; §$p<0.001$ versus young control, ‡$p<0.001$ versus young control and phagocytosis,*$p<0.001$ versus aged matched control by one-way ANOVA. Data are shown as mean $\pm$ SEM; N=9-12 per group for phagocytosis assays and N=4-8 for FcγRIII studies.

Similar to these findings, no age-dependent changes in phagocytosis by wound macrophages were observed (**Figure 11A-B**). In both young and aged mice, the frequency of FcγRIII^{hi} macrophages was reduced post-phagocytosis as compared to cells from age-matched controls (**Figure 11C**, $p<0.05$). However, there were no age-dependent differences in the frequency of total FcγRIII^{+} macrophages between young and aged mice pre- or post-phagocytosis (**Figure 11C**). Moreover, there was a reduction in the MFI of FcγRIII in aged mice compared to young regardless of exposure to pHrodo-*S. aureus* (**Figure 11D**, $p<0.05$).

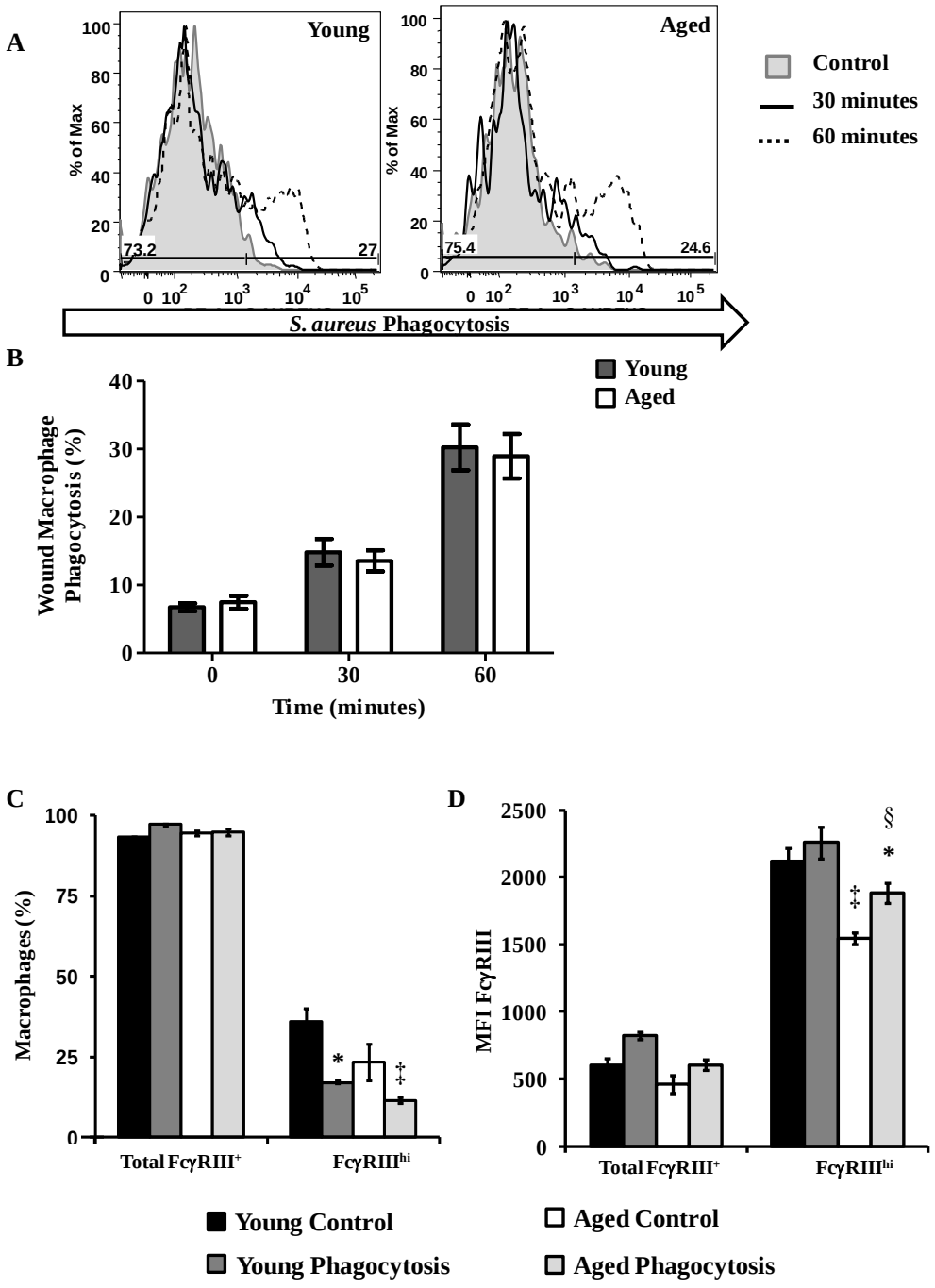


Figure 11. Wound macrophage phagocytosis of *S. aureus* and FcγRIII expression.

Wound leukocyte suspensions were allowed to phagocytose pHrodo-*S. aureus* particles for 0-60 minutes and then stained for FcγRIII expression. Phagocytosis and FcγRIII were

assayed by flow cytometry. (**A**) Representative histograms (shaded gray-4^{o}C, control; black-37^{o}C, 30 minutes; and black dotted-37^{o}C, 60 minutes) of phagocytosis by wound macrophages (F4/80^{+}Gr-1^{-}*S. aureus*$^{+}$). (**B**) Percentage of wound macrophages that phagocytosed pHrodo-*S. aureus* particles in young (gray bars) and aged (white bars) mice. Data are not significant. (**C**) Percentage of wound macrophages expressing FcγRIII in young and aged mice; *p<0.05 versus young control, ‡p<0.01 versus aged control by one-way ANOVA. (**D**) MFI of FcγRIII on wound macrophages from young and aged mice; ‡p<0.01 versus young control,*p<0.05 versus young phagocytosis, §p<0.05 versus aged control by one-way ANOVA. Data are shown as mean ± SEM; N=9-12 per group for phagocytosis assays and N=4-8 for FcγRIII studies. *p<0.05 versus young control, §p<0.05 versus young control and young phagocytosis, ‡p<0.05 versus age control.

Though age did not impact the phagocytic potential of isolated wound neutrophils or macrophages *ex vivo*, decreased FcγR expression has been shown to play a role in bactericidal activity via Rac-2 mediated formation of the NADPH oxidase complex (32, 42). To evaluate if the observed reduction in FcγRIII impaired the ability of neutrophils to kill ingested organism, isolated peripheral blood neutrophils were subjected to a modified bactericidal assay (179). Neutrophils were allowed to phagocytose opsonized pHrodo-*S. aureus* for 15 minutes, and percent killing of ingested bacteria was evaluated at 45 and 75 minutes after the end of the phagocytosis phase. No age-related differences in bactericidal activity were observed at either time point (**Figure 12**).

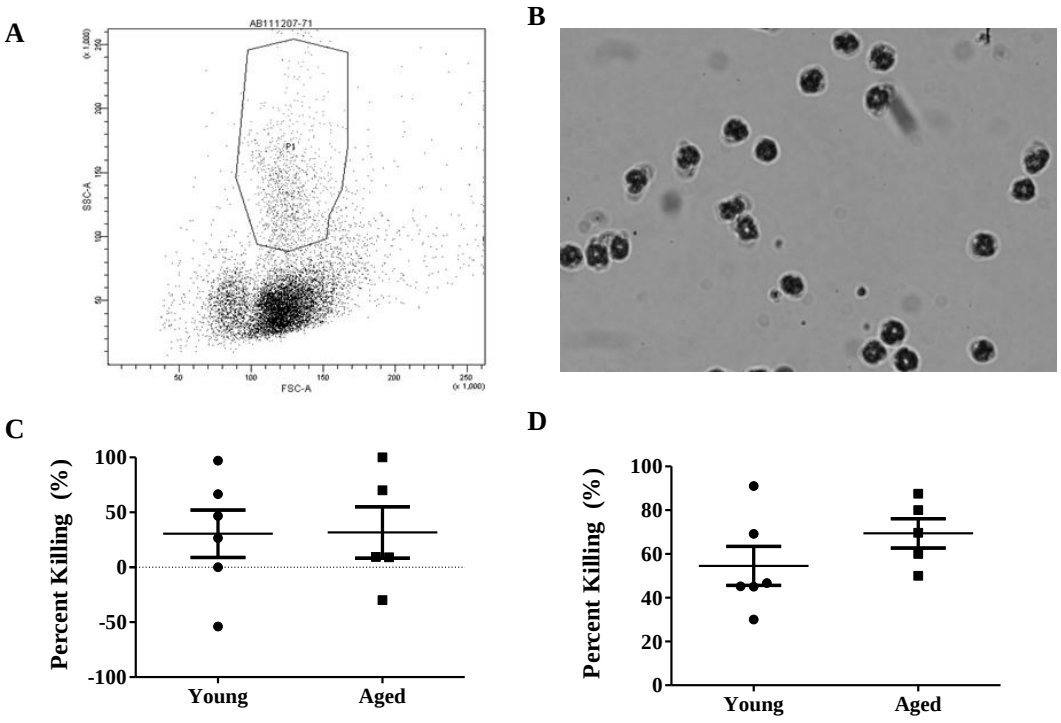


Figure 12. Bactericidal activity of peripheral blood neutrophils. Peripheral blood neutrophils were sorted from whole blood based on FSC and SSC via the FACSAria (**A**). (**B**) Cytospin of the sorted neutrophil population. hours to determine the amount of bacteria phagocytosed. Bactericidal activity (percent killing) was determined by: (number of colonies remaining at either 45 or 75 minutes/number of colonies initially present after the phagocytosis phase)*100. Bactericidal activity at 45 minutes (**C**) and 75 minutes (**D**). Data are shown as a scatter plot with mean $\pm$ SEM; N=5-6 per group. Data are not significantly different.

Peak leukocyte infiltration is attenuated in aged mice

As differences in host recognition, phagocytosis and bactericidal activity did not seem to contribute to delayed resolution of bacterial wound infection in aged animals, we hypothesized that age impairs the recruitment of leukocytes to the infected wound bed. Previous studies have documented that advanced age can alter the inflammatory cell infiltrate in non-infected wounds of aged mice (49, 50); however, the impact of cutaneous infection on leukocyte recruitment with age has not been examined. Wound leukocyte recruitment was assayed by flow cytometry at days 1-10 after injury and infection (**Figure 13**). At day 1, a similar number of wound neutrophils and macrophages were observed in young and aged mice (**Figure 13A-B**). However, at day 3, there was a reduction in neutrophil and macrophage numbers isolated from wounds from aged mice. (**Figure 13A-B**, $p<0.05$). Numbers of wound neutrophils and macrophages were comparable in young and aged mice at day 7, despite persistent infection in aged animals. No difference in T cell recruitment was noted at any time point (**Figure 13C**). The delay in neutrophil and macrophage recruitment at day 3 in aged animals may impair early bacterial clearance while the inability to recruit elevated numbers of leukocytes in aged mice at later time points may contribute to the persistent infection observed in these animals. Moreover, this altered leukocyte recruitment may perturb wound healing kinetics, delaying transition from the inflammatory to the proliferative phase and ultimately impairing wound closure and restoration of the dermal matrix.

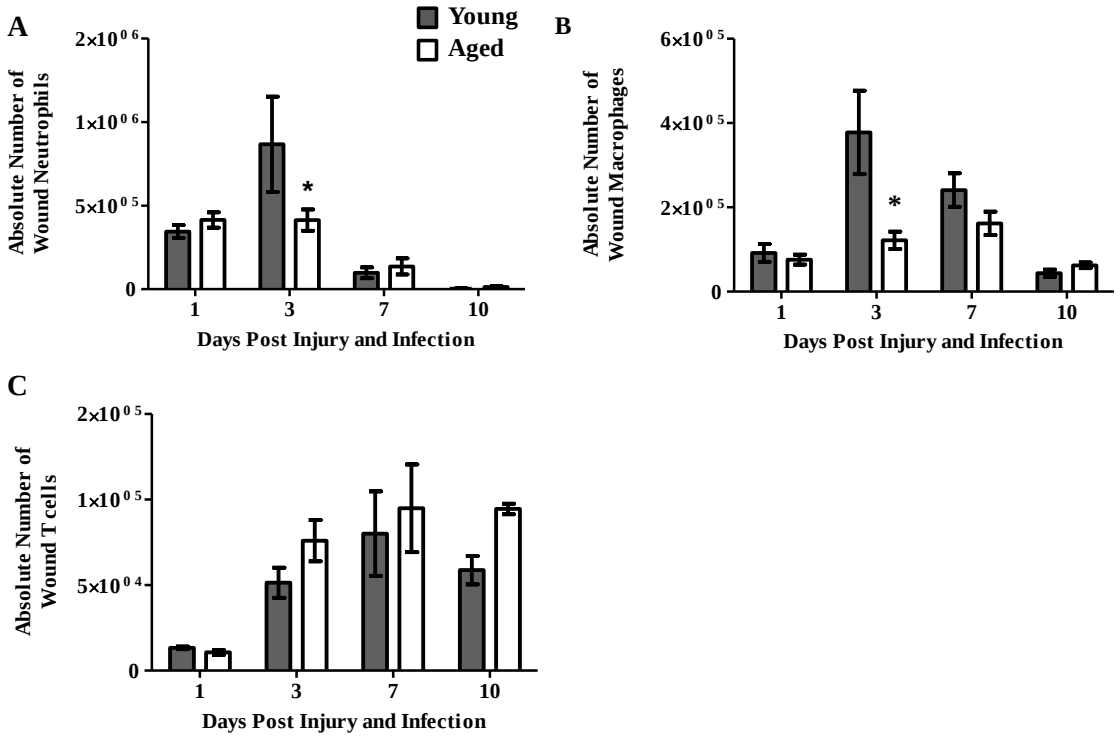


Figure 13. Time course of cutaneous leukocyte accumulation following injury and infection. Leukocyte infiltration to the wound and surrounding tissue area of young (gray bars) and aged (white bars) was assessed by flow cytometry at days 1 through 10 post cutaneous wound infection. (**A**) Gating strategy for wound neutrophils ($F4/80^{-}/Gr\text{-}1^{+}$), wound macrophages ($F4/80^{+}/Gr\text{-}1^{-}$) and wound T cells ($CD3^{+}$) by flow cytometry. (**B**) Absolute number of wound neutrophils; *$p<0.01$ compared to young at same time point by two-way ANOVA. Data are shown as mean ± SEM; N=8-19 per group at days 1-7, N=3-6 at day 10. (**C**) Absolute number of wound macrophages. Data are shown as mean ± SEM; N=8-19 per group at days 1-7, N=3-6 at day 10. (**D**) Absolute number of wound T cells. Data are shown as mean ± SEM; N=3-9 per group. Data are not significantly different by two-way ANOVA.

Enhanced chemokine secretion in wounds from aged animals

Important to neutrophil and macrophage recruitment are the chemotatic stimuli generated following tissue injury by keratinocyte and resident tissue leukocytes (128, 188). To determine if the decreased neutrophil and macrophage numbers were due to reduced chemoattractant production, we evaluated the wound chemokine milieu. Interestingly, at day 1 following injury and infection, wounds from aged mice had elevated levels of KC and MIP-2, potent neutrophil chemokines and the murine homologs of human IL-8 (**Figure 14A-B**, $p<0.05$). Furthermore, wounds from aged mice had increased levels of MCP-1, a chemokine that helps recruit and differentiate circulating monocytes (**Figure 14C**, $p<0.05$). However, these elevated levels of neutrophil and macrophage chemokines were associated with similar neutrophil and macrophage recruitment at day 1. At day 3, when neutrophil and macrophage numbers were significantly reduced in aged animals, levels of these three chemotatic mediators were similar in young and aged mice (**Figure 14**, $p<0.05$). These data suggest that aged mice may require a stronger chemotatic stimulus at the site of injury and infection to generate recruitment of a similar number of infiltrating leukocytes.

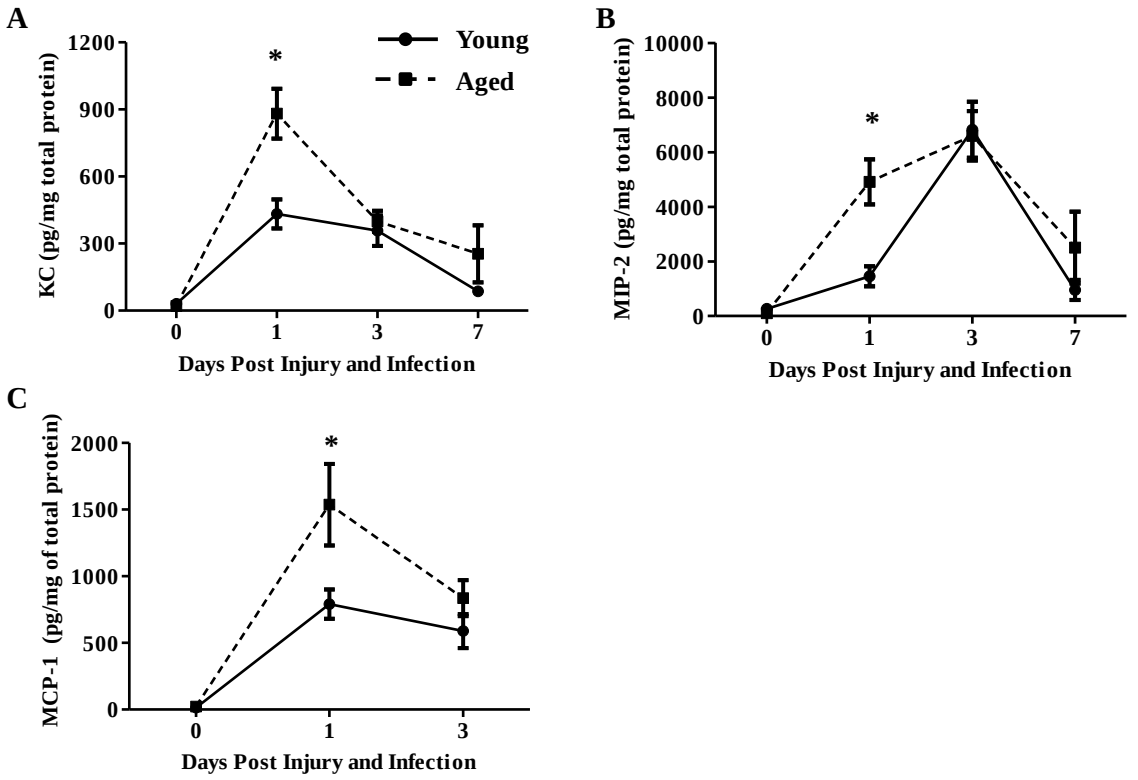


Figure 14. Neutrophil and macrophage chemokine levels in wound homogenates. Neutrophil and macrophage chemokine levels in wounds of young (black circles and solid line) and aged (black squares and dotted line) mice present at the site of wound infection were measured by ELISA. (**A**) KC, *$p<0.0001$ compared to young at same time point by two-way ANOVA. (**B**) Macrophage inflammatory protein-2 (MIP-2), *$p<0.05$ compared to young at same time point by two-way ANOVA. (**C**) Macrophage chemoattractant protein-1 (MCP-1), *$p<0.05$ compared to young at same time point by two-way ANOVA. Data are shown as mean + SEM; N=8-22 per group (days 0-3) and N=3-6 per group (day 7).

Elevated CXCR2 expression in peripheral blood neutrophils of aged animals following injury

KC and MIP-2 are ligands for the chemotatic receptor CXCR2 present on circulating neutrophils (189). Ligation of CXCR2 results in upregulation of selectins and adhesion molecules which allow neutrophils to roll, adhere and subsequently transmigrate across endothelial walls to reach the site of injury or infection (26, 28, 190, 191). Considering a strong chemotatic stimulus was present at the wound site in aged animals, we examined the peripheral neutrophil pool to determine if age-dependent differences in CXCR2 expression could account for reduced leukocyte recruitment with age. Following injury and infection at days 1 and 3, the frequency of neutrophils in circulation was elevated in aged mice compared to young mice at both time points (**Figure 15A-B**, $p<0.05$). There was no difference in the frequency of $CXCR2^{+}$ neutrophils between young and aged mice prior to or following injury (**Figure 15C**). Following cutaneous wound infection, downregulation of CXCR2 expression was observed in mice of both age groups, however the MFI of CXCR2 on neutrophils from aged mice was significantly elevated (**Figure 15D**). This elevation persisted out to day 3, where CXCR2 expression remained increased in aged mice relative to young mice. These data suggest that mediators downstream of CXCR2 may contribute to decreased neutrophil recruitment to the wound site in aged animals.

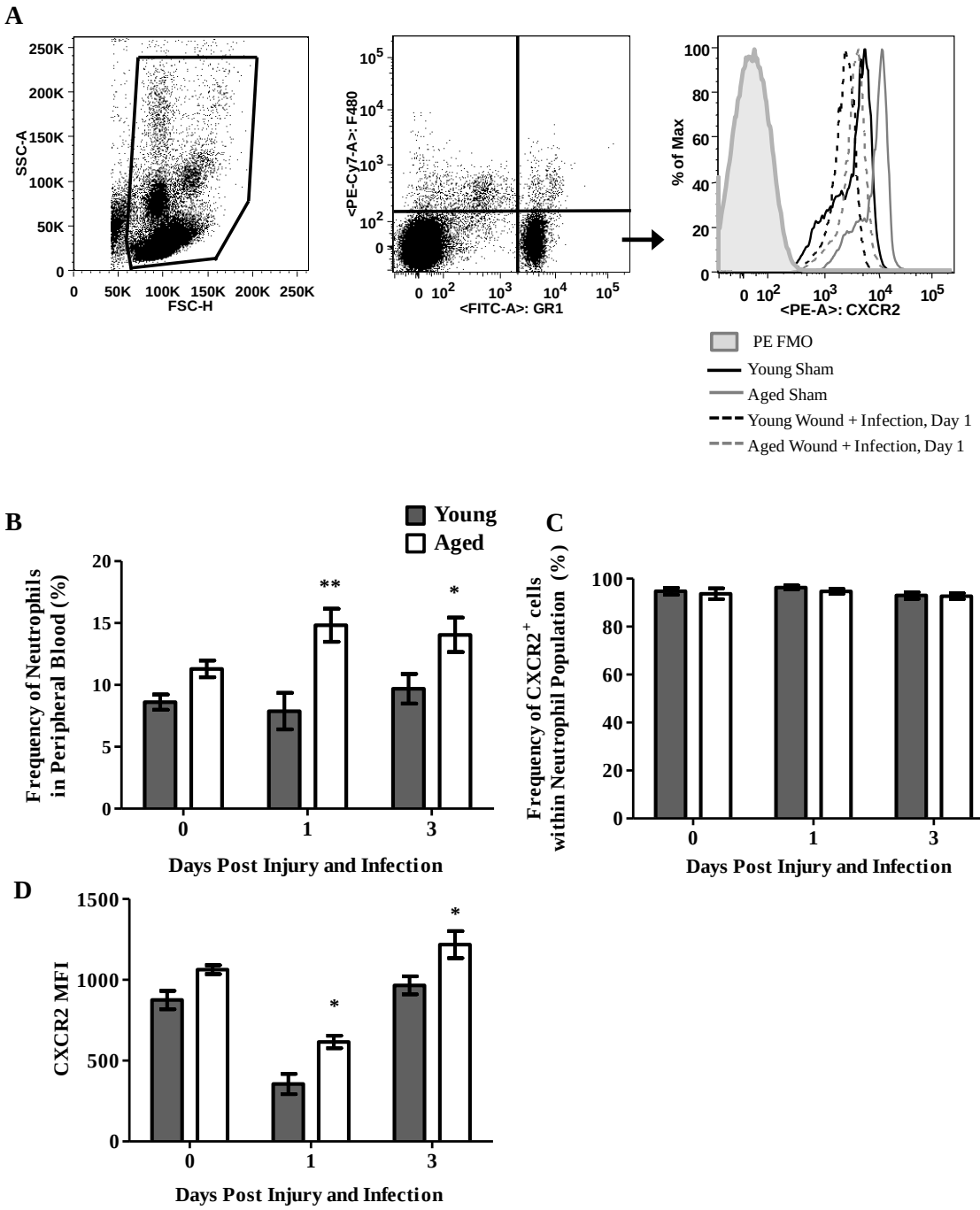


Figure 15. Peripheral blood neutrophil frequency and chemokine receptor expression. Whole blood was analyzed by flow cytometry to determine the percentage of circulating neutrophils and expression of the chemotatic receptor CXCR2. (**A**) Gating strategy for neutrophils in whole blood by flow cytometry. (**B**) Percentage of circulating

neutrophils in peripheral blood in young (gray bars) and aged (white bars) mice. (**C**) Frequency of $CXCR2^{+}$ neutrophils in peripheral blood. (D). Expression of CXCR2 on peripheral blood neutrophils. Data shown as mean $\pm$ SEM, N=9-18, *$p<0.05$ and **$p<0.001$ versuss young at same time point by two-way ANOVA.

Aging impairs in vivo chemotaxis to dorsal cutaneous skin

Reduced neutrophil accumulation in spite of increased CXCR2 expression coupled with a comparable chemotactic stimulus in aged mice as compared to young suggests that aging may contribute to a neutrophil migratory defect. Previously, our laboratory has demonstrated that peripheral blood neutrophils from aged animals demonstrate a basal hyperchemokinesis with a reduced directional migration toward a chemotatic stimulus *in vitro* (60). We extended these findings to determine if reduced neutrophil numbers in cutaneous infected wounds in aged animals may be due to a chemotatic deficiency *in vivo*. Following *s.c.* injection of 100 pg of KC, neutrophil recruitment to the dorsal skin in aged mice was reduced as compared to young (**Figure 16**, $p<0.05$). To determine if this could be overcome by higher doses of a chemotactic agent, we evaluated neutrophil recruitment after injection of 1,000 pg of KC. At a ten-fold higher dose, aged mice still had impaired neutrophil recruitment as compared to their younger counterparts given the same dose (**Figure 16**, $p<0.05$). However, neutrophil recruitment in aged mice at a dose of 1000 pg was similar to that observed in young mice at a dose of 100 pg. These findings parallel our results observed following cutaneous wound infection in aged mice, suggesting that a higher chemotatic stimulus may be required to mediate a similar chemotatic response with advanced aged. Together, these data suggest that reduced neutrophil recruitment to site of cutaneous wound infection may be due to impaired chemotaxis with advanced age, independent of the level of chemoattractant stimuli or CXCR2 expression.

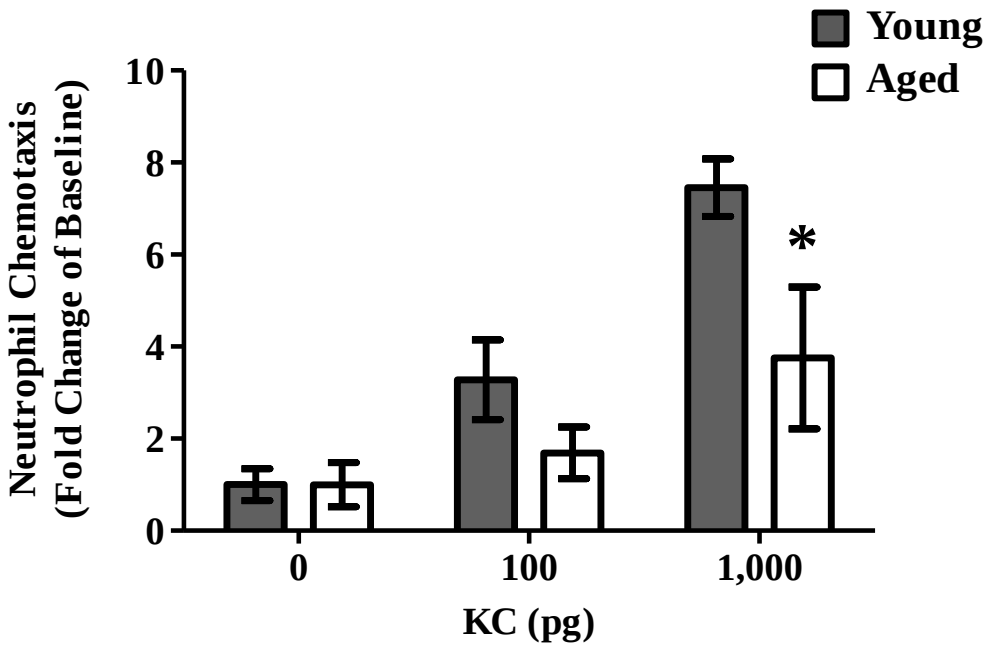


Figure 16. *In vivo* neutrophil chemotaxis in response to KC. Young and aged mice were injected *s.c.* with increasing doses of KC (0, 100 and 1000 pg) and neutrophil recruitment to the skin was examined by flow cytometry. Data are shown as mean ± SEM, N=3-6 per group, *p<0.05 versuss young at same time point by two-way ANOVA.

Summary

We developed a murine model of *S. aureus* cutaneous wound infection that allows for assessment of age-dependent differences in the innate immune response. In aged animals, we observed elevated levels of bacteria in wounds at early time points with active infection to 10 day in aged animals. Our data is in concert will clinical and murine studies that demonstrate aging is associated with elevated rates of infection (121, 159, 174, 192). Furthermore, we are the first to demonstrate that wound closure was delayed in aged animals as compared to young following *S. aureus* wound infection. Again, these studies are in-line with aging mouse studies that report decreased wound healing in aseptic wound models (49, 63).

We evaluated several parameters of the innate immune response to determine what may be contributing to these age-associated differences in bacterial burden and wound healing. First, we examine TLR2, a PRR responsible for initiating immune responses to Gram-positive bacteria. While we observed a reduction in TLR2 expression in resident tissue macrophages from uninjured skin in aged mice, these age-related differences were not observed in infiltrating leukocytes at days 1 and 3 after injury and infection. These data suggest that diminished TLR2 levels in resident macrophages may play a role in heightened susceptibility to infection with age; however, alterations in TLR2 likely do not contribute to delayed resolution of wound infection in aged animals. While some groups have reported that aging negatively impacts a variety of TLRs (74), others demonstrate no impact of age on TLR expression in a variety of cell subsets (74, 101, 192). These reports suggest that the effect of age on TLR expression or function

may be cell type or model dependent, and future work to clarify the impact of age on TLR function needs to be conducted in clinical relevant models.

Another critical neutrophil and macrophage function is phagocytosis and killing of foreign pathogens. Previous studies have reported that aging negatively impacts neutrophil and macrophage phagocytosis (46, 50, 120, 185, 186), though few have evaluated the phagocytic potential of leukocytes following recruitment in response to tissue injury. In our model of wound infection, no age-dependent differences in wound neutrophil or macrophage phagocytosis were noted. Downstream of FcγR activation, the small GTPase Rac2 plays an essential role in assembly and recruitment of the NADPH oxidase complex to the phagosome to mediate bactericidal activity (32, 42). Despite reduction of FcγR levels in aged animals, no difference in bactericidal activity was observed. While other Fc receptors and phagocytic pathways were not examined, our data imply that age does not impair Fcγ-mediated phagocytosis in wound neutrophils or macrophages.

Most appreciably, we observed delayed recruitment of neutrophils and macrophages to the site of wound infection in aged animals as compared to young. Alterations in infiltration of these cells can impact several facets of wound healing. For example, reduced phagocyte recruitment may impair bacterial clearance and promote infectious spread (193). Moreover, studies in aging models have suggested requirement for neutrophils in successful wound healing with advanced age (49). Increasing neutrophil numbers via G-CSF administration enhanced rates of wound closure in aged mice to those observed in young mice (49). In the context of an infected wound, the need for neutrophil infiltration becomes increasingly paramount as patients with reduced

neutrophil function or numbers are at a heightened risk for chronic and repeated infections with catalase-positive bacteria, like *S. aureus* (43, 184, 194). Thus, the decreased absolute numbers of neutrophils and macrophages in our model may not only contribute to bacterial colonization differences observed with age, but may also play a role in delayed wound resolution in aged animals.

As mentioned previously, this weakened neutrophil response may impair resolution of wound infection in aged mice. Recently, we demonstrated that peripheral blood neutrophils from unmanipulated young and aged mice have a basal hyperchemokinesis but lack directional migration *ex vivo* (60). These studies extend these findings and show a reduced migratory response to in vivo, *s.c.* KC in aged mice relative to young. Interestingly, this impaired recruitment to cutaneous tissues was observed in spite of elevated CXCR2 expression on circulating neutrophils from aged animals. Together, these data indicate that signaling downstream of CXCR2 may be altered with age. Future work to determine the mechanism associated with impaired chemotaxis with age may translate into therapeutic modalities which can improve resolution of wound infection in aged animals.

CHAPTER 4

G-CSF ENHANCES RESOLUTION OF *STAPHYLOCOCCUS AUREUS* WOUND INFECTION IN AN AGE-DEPENDENT MANNER

Abstract

This study tested the hypothesis that heightened bacterial colonization and delayed wound closure in aged mice could be attenuated by G-CSF treatment. Previously, we reported that aged mice had elevated bacterial levels, protracted wound closure and reduced wound neutrophil accumulation following *S. aureus* wound infection relative to young mice. In aseptic wound models, G-CSF treatment improved wound closure in aged mice to rates observed in young mice. Given these data, our objective was to determine if G-CSF could restore age-associated differences in wound bacterial burden and closure and increase wound neutrophil recruitment. Young (3-4 month) and aged (18-20 month) BALB/c mice received three dorsal, subcutaneous injections of G-CSF (250 pg/50 μl/injection) or saline control (50 μl/injection) 30 minutes after wound infection. Mice were sacrificed at days 3 and 7 post wound infection and bacterial colonization, wound size, wound leukocyte accumulation and peripheral blood were evaluated. At days 3 and 7 after wound infection, bacterial colonization was significantly reduced in G-CSF-treated aged mice to levels observed in saline-treated young animals. Wound size was reduced in G-CSF-treated aged animals, with no affect on wound size in G-CSF-treated

young mice. Local G-CSF treatment significantly enhanced neutrophil wound accumulation in aged mice at day 3 and 7, whereas there was no G-CSF-induced change in young mice. G-CSF treatment did not significantly impact macrophage or T cell infiltration at days 3 and 7 in young or aged mice. These data demonstrate that G-CSF enhances bacterial clearance and wound closure in an age-dependent manner. Moreover, G-CSF may be of therapeutic potential in the setting of post-operative wound infection or chronic, non-healing wounds in elderly patients.

Introduction

Chronic, non-healing wounds are estimated to account for over 25 billion dollars in US health care costs (151). The failure to effectively heal wounds is often compounded by co-morbidities, such as diabetes or obesity. Another major patient population afflicted with chronic wounds are the elderly (119, 151, 159). With advanced age, a decline in immune function elevates rates of wound infection and further impairs wound closure in these patients (64, 160, 161). In particular, *S. aureus* infection accounts for 30% of surgical site infections in healthy, non-aged patients (159). In the elderly, *S. aureus* infection jumps to 50%, suggesting a proclivity of this Gram-positive bacterium for the immunocompromised (159). This increased predilection for infectious complications raises additional public health concerns, such as prolonged hospital stays, risk for septic complications or infectious spread in long-term care accommodations. As we continue to see a precipitous rise in the aged population, the number of total patients affected by non-healing wounds is expected to rise from the estimated 6.5 million (151). Moreover, despite investment in antibiotic development for multi-drug resistant bacterial strains,

these bacteria continually evolve to evade our treatment options (195). New therapeutic targets that harness the host immune system to help eradicate infection and promote efficient wound healing will be necessary to decrease the public health cost due to chronic wounds and wound infection (184).

Despite the knowledge that aging impairs wound healing, little has been done to determine how the early innate immune response is altered with age in the setting of wound infection. Models of aging and wound healing have shown alteration in the innate immune response during the early inflammatory phased of wound healing, as well as perturbations in mediators of the proliferative and remodeling stages (50, 63, 196-199). To evaluate how age negatively impacts the innate immune response to cutaneous wound infection, we previously developed a model of cutaneous wound injury and *S. aureus* infection in young and aged BALB/c mice. In this model, aged mice had heightened levels of bacterial colonization and delayed wound closure as compared to young animals. These findings were associated with reduced neutrophil recruitment to the wound site, despite elevated chemokine levels and adequate peripheral blood neutrophil CXCR2 expression. Moreover, we saw diminished neutrophil chemotaxis to the skin following subcutaneous injections of the murine neutrophil chemoattractant KC. Others have demonstrated that addition of G-CSF to their model of aseptic wound healing enhanced wound closure in aged animals (49), and that G-CSF can restore defects in age-associated neutrophil chemotaxis (200). Herein, we are the first to demonstrate that local administration of G-CSF reduced bacterial colonization and enhanced wound closure in aged animals via increased neutrophil recruitment at early time points. These data suggest

that local G-CSF treatment may serve as a potential therapeutic intervention in elderly patients with wound infections.

Materials and Methods

Animal Model of Cutaneous Injury and Infection

Young (3-4 month) and aged (18-20 month) BALB/c mice (Charles River/NIA, Kingston Facility, Stony Ridge, NY) were used to determine if local, subcutaneous (*s.c.*) G-CSF treatment could restore age-dependent differences to *S. aureus* wound infection (**Figure 17**). All animal studies were approved and performed with strict accordance to the regulations established by the Loyola University Chicago Animal Care and Use Committee. Following acclimation at Loyola's Animal Care Facility, young and aged mice were subjected to dorsal excisional cutaneous injury as previously described (175). Briefly, mice were given 10 mg/kg xylazine and 100 mg/kg ketamine intraperitoneal *(i.p.)* followed by *i.p*. saline to ensure systemic distribution of the anesthetic. Dorsal skin of the mice was placed over a hockey puck, and mice were subjected to 3 sets of 2, 3 mm dermal punch wounds (Acuderm, Ft. Lauderdale, FL) for a total of 6 dorsal full-thickness cutaneous wounds. Immediately after injury, mice received ~10^3 CFU of *S. aureus*/10 μL of saline and were returned to their cages on heating pads. At 30 minutes after injury and infection, animals received three *s.c*. G-CSF injections (250 pg/50 μl/injection; R&D Systems, Minneapolis, MN) or saline vehicle injections (50 μl/injection) in the dorsal midline in between each set of wounds. As sepsis is known to negative impact wound healing, a low bacterial inoculum was used to limit the risk of systemic dissemination of bacteria (176). *S. aureus* Newman strain was grown overnight in TSB at 37^{o}C under

constant agitation. The next day, 1 mL of the overnight culture was resuspended in 2mL fresh TSB and incubated at 37^{o}C for 2 hours to ensure mid-logarithmic growth at the time of application to cutaneous wounds. Absorbance at 600 nm was used to determine the bacterial concentration (CFU/mL) and the final inoculum was confirmed by back-plating on MSA (BD Diagnostics, Sparks, MD).

Mice were sacrificed at days 3 and 7 after injury and infection. The pelt was removed and photographed to measure wound size as described below. A larger 5 mm punch biopsy was used to remove the 3 mm wounds. Wounds were examined for bacterial colonization and flow cytometric analysis of wound immune infiltrate (Brubaker *et. al* 2012, manuscript submitted). Heparinized whole blood was obtained via cardiac puncture for flow cytometric analysis (Brubaker *et. al* 2012, manuscript submitted).

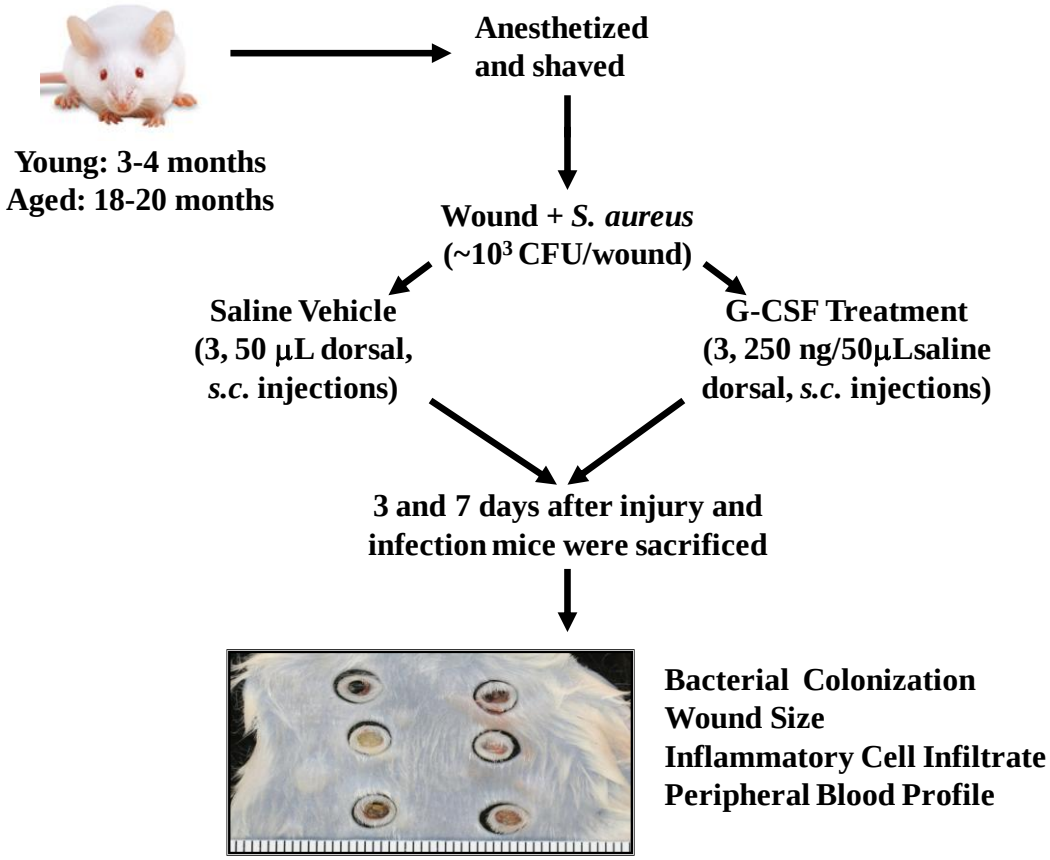


Figure 17. Model of *S. aureus* cutaneous wound infection +/- G-CSF treatment in young and aged mice. Young (3-4 month) and aged (18-20 month) BALB/c mice were anesthetized and their dorsum shaved. Mice then received 6, 3 mm dorsal punch wound followed by ~10^3 CFU of *S. aureus* per wound. Saline or G-CSF was administered 30 minutes after injury and infection. Mice were sacrificed at days 3 and 7. Wounds were assessed for bacterial colonization, wound size and inflammatory cell infiltrate. The peripheral blood was harvested for evaluation by flow cytometry.

Bacterial Colonization

One skin wound was homogenized in 1 mL sterile PBS (Gibco, Grand Island, NY) and 10-fold serial dilutions to 10^6 were plated on MSA plates (BD Diagnostics, Sparks, MD). Plates were incubated for 24-48 hours at 37^{o}C and colonies were counted to determine levels of bacterial colonization (Brubaker *et. al* 2012, manuscript submitted).

Wound Size

Wound size was evaluated by digital photography and image analysis as previously described (175). At days 3 and 7 wounds were photographed with a Canon EOS SLR digital camera. Each pelt was placed flat on a hockey puck and photographed at a fixed distance of 20 cm with a ruler placed within the frame of each photograph. Photoshop 7.0 (Adobe Systems Inc., San Jose, CA) was used to determine the number of pixels in the open wound area using the magic wand tool, with a tolerance setting of 60 and zoom at 100%. Separate animals were sacrificed immediately following wound injury and wound size was determined to represent day 0. Wound areas at each time point were compared with day 0 wounds: (pixels at days1-10/pixels at day 0)^{x}100 were used to determine the percent open wound area at each time point.

Skin Cell Isolation and Flow Cytometry

Single cell suspensions of wound cells for flow cytometry were generated as previously described (177). At days 3 and 7 after injury and infection, animals were euthanized, pelts removed and wounds excised using a 5 mm punch biopsy. Two diced wounds were incubated overnight at 4^{o}C in RPMI 1640 culture media (Gibco, Grand

Island, NY) containing 10% FBS (Hyclone, Logan, UT), 2 mM L-glutamine (Gibco, Grand Island, NY), 1% penicillin/streptomycin (Gibco, Grand Island, NY), 2 mg gentamycin sulfate (Mediatech Inc, Manassas, VA) and 0.3 mg dispase II (Roche Diagnostics, Indianapolis, IN). Tissue pieces were then removed and subjected to further enzymatic digestion with 1 mg collagenase from *Clostridium histolyticum* type 1A (Sigma-Aldrich, St. Louis, MO), 1.2 mg DNAse I from bovine pancreas Grade II (Roche Diagnostics, Indianapolis, IN), 1 mg hyaluronidase from bovine testes type 1-S (Sigma-Aldrich, St. Louis, MO), in RPMI 1640 with 5% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, gentamycin and magnesium chloride hexahydtrate for 2 hours at 37^{o}C. After two hours, these solutions were combined and adherent cells were treated with Accutase (eBioscience, San Diego, CA) for 8 minutes at 37^{o}C followed by vigorous pipetting. Single cells suspensions were filtered through at 70 μm filter to remove debris. Cells were washed and adjusted to 1x10^{6}/mL. Wound cells were blocked for 20 minutes with FcBlock (anti-CD16/CD32, eBioscience) and rat IgG (Jackson ImmunoResearch, West Grove, PA), and then stained with PE-Cy7-F4/80 (eBioscience, San Diego, CA), FITC-Gr-1 (eBioscience, San Diego, CA) and APC-CD3 (eBioscience, San Diego, CA). Cells were washed twice and then resuspended in flow buffer (1% BSA (Sigma-Aldrich, St. Louis, MO), 0.1% sodium azide and 2mM EDTA in PBS). Events were collected on the FACS LSR Fortessa (BD Biosciences, San Jose, CA) and data were analyzed by FlowJo Software (Tree Star Inc, Ashland, OR). Fluorescence minus one control staining and single color controls were used to determine positive staining. F4/80^{+}Gr-1^{-} cells were considered macrophages, F4/80^{-}Gr-1^{+} cells were considered neutrophils and CD3^{+} cells were considered T cells.

Blood Flow Cytometry

Heparinized whole blood was collected and stained per the manufacture's protocol (Leinco Technologies, St. Louis, MO). 100 μL whole blood was blocked for 20 minutes with FcBlock and rat IgG and then stained with PE-Cy7-F4/80, FITC-Gr-1 and PE-CXCR2 (R&D Systems, Minneapolis, MN). Erythrocytes were then lysed with 2 mL Easy Lyse Solution (Leinco Technologies, St. Louis, MO) for 11.5 minutes. Lysis was stopped by addition of 2 mL ice cold Wash Buffer (Leinco Technologies, St. Louis, MO) and placement on ice. Cells were washed twice and resuspended in 500 μL flow buffer. Events were collected on the FACS LSR Fortessa (BD Biosciences, San Jose, CA) and data were analyzed by FlowJo Software (Tree Star Inc, Ashland, OR). Fluorescence minus one control staining and single color controls were used to determine positive staining. $F4/80^{-}Gr\text{-}1^{+}$ cells were considered peripheral blood neutrophils.

Statistical analysis

Data are shown as mean ± SEM of each group. Data were analyzed by Student's *t* test or one-way ANOVA with Tukey's post-hoc tests where appropriate using GraphPad Prism 5 (GraphPad, La Jolla, CA). A value of $p \leq 0.05$ was considered significant.

Results

Local G-CSF treatment improves resolution of bacterial infection and wound closure in aged mice

Previously, we demonstrated that aged mice exhibited elevated bacterial colonization following cutaneous *S. aureus* wound infection that correlated with reduced neutrophil recruitment (Brubaker *et. al* 2012, manuscript submitted). G-CSF has been shown to enhance neutrophil recruitment and improve wound closure in aged animals (49), as well as restore age-associated chemotatic defects (200). Thus, we chose to investigate if G-CSF could restore bacterial clearance in aged mice by enhancing wound leukocyte recruitment. Following G-CSF administration, we saw a 75% decrease in bacterial colonization at day 3 in aged mice as compared to aged, saline control animals (**Figure 18A**, $p<0.05$). At day 3, G-CSF did not affect wound bacterial levels in young mice. These differences in wound *S. aureus* colonization persisted out to day 7 with a 97% reduction in bacterial growth in aged animals administered G-CSF compared to aged-saline control (**Figure 18B**, $p<0.05$). While a mild elevation in day 7 wound bacterial levels in G-CSF-treated young mice was observed, this difference was not statistically significant from young saline.

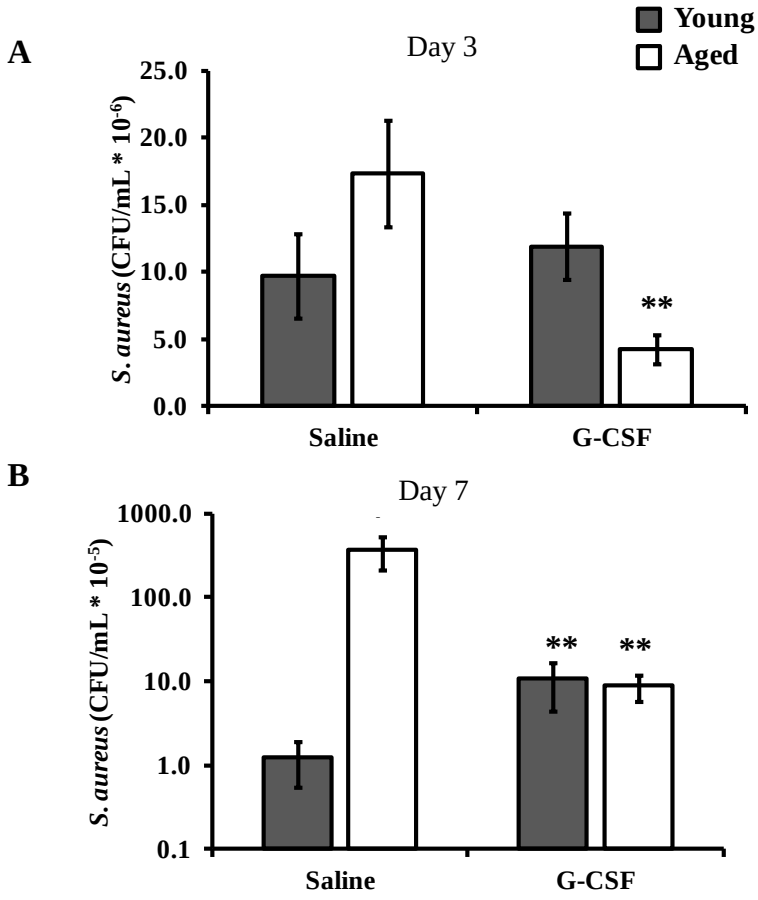


Figure 18. Local G-CSF treatment reduces bacterial colonization in aged mice.

Young BALB/c (3-4 month, gray bars) and aged BALB/c (18-20 month, white bars) received six, 3 mm dorsal cutaneous wounds followed by ~10^3 CFU *S. aureus*/wound. 30 minutes after injury and infection, mice either received *s.c.* G-CSF injections (250 ng/50 μl/injection) or saline vehicle injections (50 μl/injection). At days 3 (**A**) and 7 (**B**), mice were sacrificed and bacterial colonization was determined by growth on MSA plates. Data are shown as mean ± SEM, *$p<0.05$ compared to young saline and **$p<0.01$ compared to aged saline by one-way ANOVA; N=7-14 per group.

In concert with these data, we observed that local injection of G-CSF enhanced wound closure in aged animals at day 3 compared to aged, saline controls (**Figure 19A+B**, $p<0.05$). At day 7, differences in wound size observed between saline treated young and aged mice was ameliorated following G-CSF treatment (**Figure 19A+C**, $p<0.05$). G-CSF treatment did not affect that rate of wound closure in young mice at day 3 or 7.

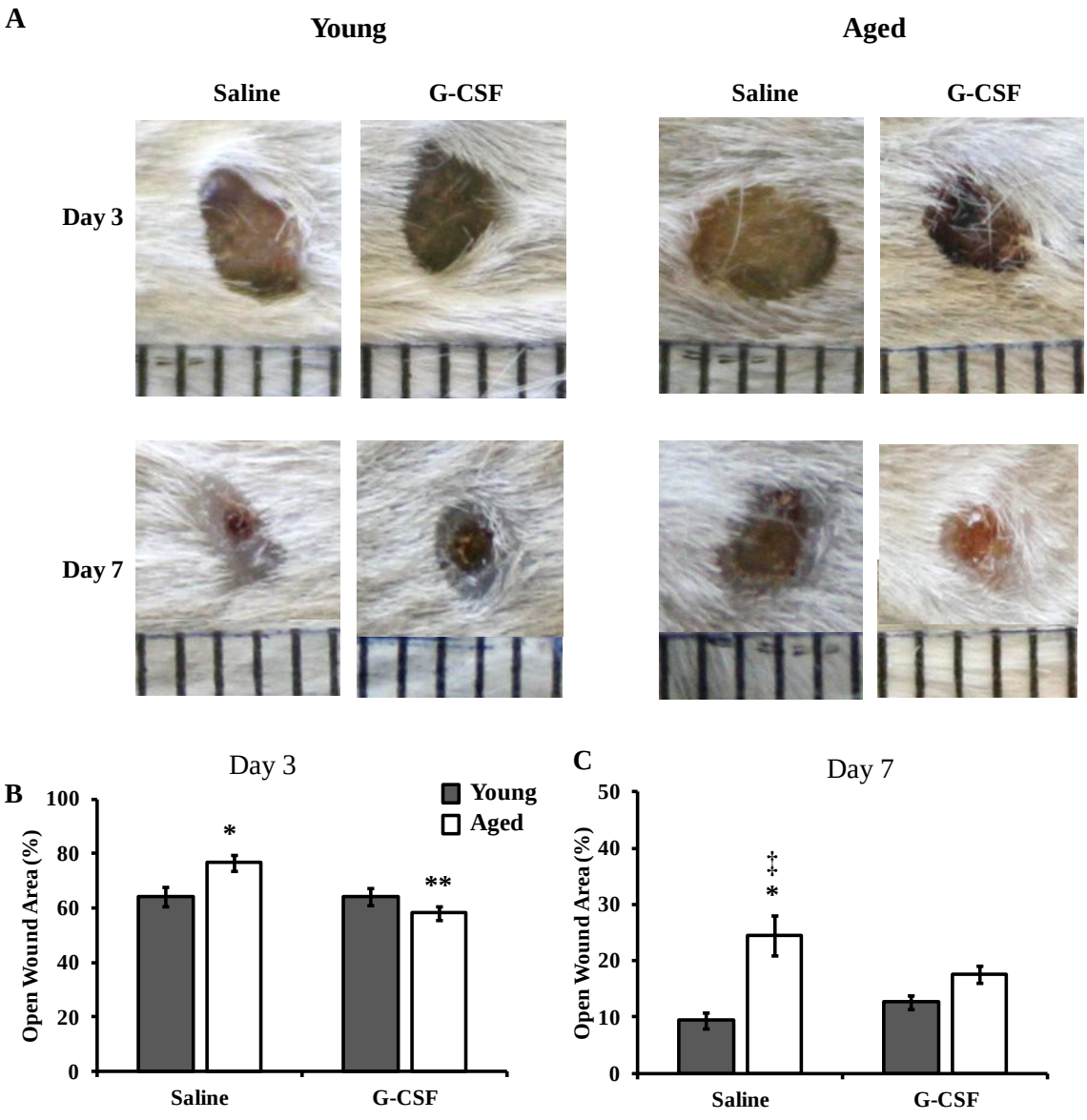


Figure 19. Treatment with G-CSF abrogates age-associated deficits in wound closure. (**A**) Representative images of wound from young (left panel) and aged (right panel) mice at days 3 (top panel) and 7 (bottom panel) after *S. aureus* wound infection with or without G-CSF treatment. Wound size expressed as percent open wound area relative to time zero at days 3 (**B**) and 7 (**C**) after cutaneous wound infection in young (gray bars) and aged (white bars) mice. Data are shown as mean ± SEM, *$p<0.05$ compared to young saline, **$p<0.01$ compared to aged saline and ‡$p<0.05$ compared to young G-CSF by one-way ANOVA; N=7-14 per group.

Wound neutrophil recruitment in aged animals in enhanced by local G-CSF treatment

Given that G-CSF restored bacterial clearance and wound closure in aged mice, we sought to examine if this correlated with enhanced leukocyte recruitment to the wound bed (**Figure 20A**). At day 3 following injury and infection, saline-treated aged mice had reduced absolute numbers of neutrophil and macrophage accumulation compared to young, saline treated mice (**Figure 20B+C**, $p<0.5$). Treatment with G-CSF increased neutrophil numbers in aged mice in respect to aged, saline treated animals (**Figure 20B**, $p<0.05$) and neutrophil recruitment was no longer different from young mice. No differences in neutrophil accumulation in young mice administered saline or G-CSF were observed. G-CSF treatment did not impact macrophage recruitment at day 3 in aged animals (**Figure 20C**). While a mild reduction in macrophage recruitment in young animals given G-CSF was observed, these differences were not significant. At day 7, G-CSF treatment resulted in protracted neutrophil accumulation of wound in aged animals as compared to all other groups (**Figure 20D**). This prolonged neutrophil infiltration may contribute to the reduced bacterial colonization observed in aged animals. No difference between wound macrophage accumulation was observed in saline- or G-CSF-treated young and aged mice (**Figure 20E**). Wound T cell recruitment at day 3 and 7 was similar between young and aged mice, and these numbers were not significantly affected by treatment with G-CSF (**Table II**).

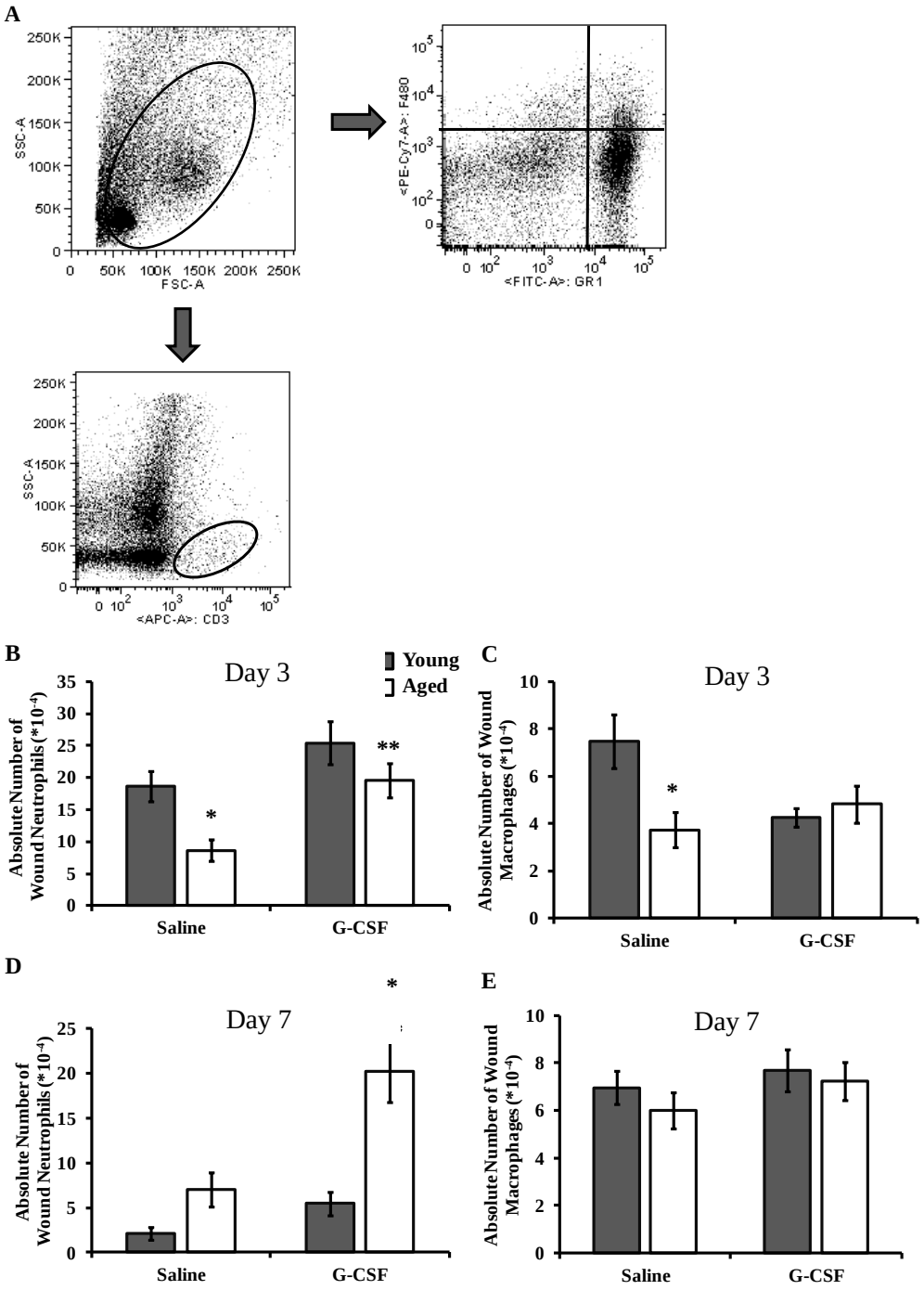


Figure 20. Neutrophil accumulation is enhanced in aged mice following G-CSF treatment. Leukocyte infiltration to the wound and surrounding tissue area was assessed by flow cytometry at days 3 and 7 following cutaneous wound infection and G-CSF treatment. (**A**) Gating strategy for wound neutrophils (F4/80$^-$/Gr-1$^+$), wound macrophages

($F4/80^+/Gr\text{-}1^-$) and wound T cells ($CD3^+$) by flow cytometry. Absolute number of wound neutrophils and macrophages at day 3 (**B**+**C**, respectively) and day 7 (**D**+**E**, respectively). Data are shown as mean ± SEM, *$p<0.05$ compared to young saline, **$p<0.01$ compared to aged saline and ‡$p<0.05$ compared to young G-CSF by one-way ANOVA; N=7-14 per group.

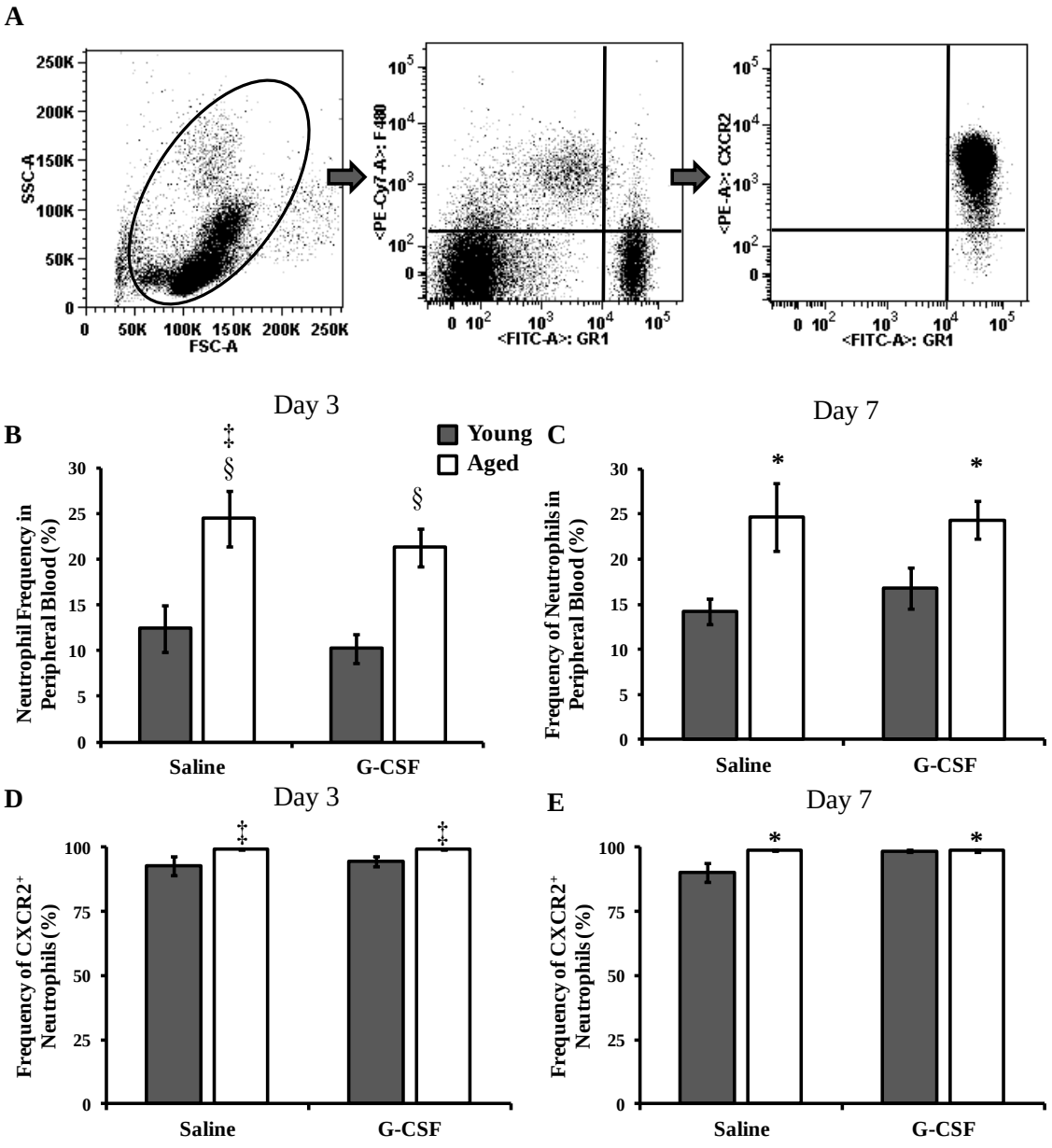
Table II. Wound T cell numbers in saline and G-CSF treated mice.

	Saline ($CD3^+$)		**G-CSF ($CD3^+$)**	
	Day 3	**Day 7**	**Day 3**	**Day 7**
Young	25194.6 ± 6141.9	23790.1 ± 2568.1	23721.5 ± 2247.4	26215.6 ± 3371.0
Aged	26659.8 ± 1563.8	30960.4 ± 5153.6	36451.4 ± 4798.5	39230.1 ± 4814.8

No differences were observed in number of infiltrating T cells ($CD3^+$) at days 3 and 7 after injury and infection between young and aged mice. T cell numbers were not significantly affected by treatment with G-CSF. Data are shown as mean ± SEM; N=7-14 per group. Data are not significantly different by one-way ANOVA.

No-age dependent effects of local G-CSF treatment on circulating leukocytes

To eliminate the possibility that local, *s.c.* G-CSF was inducing changes in the peripheral blood pool, we evaluated the frequency of circulating neutrophils in young and aged mice given saline or G-CSF injections (**Figure 21**). As reported previously (Brubaker *et. al* 2012, manuscript submitted), aged mice exhibited heightened levels of circulating neutrophils at day 3 compared young mice (**Figure 21B**, $p<0.5$), and this was not altered by G-CSF administration. This age-dependent elevation in the frequency of peripheral blood neutrophils persisted out to day 7 (**Figure 21C**, $p<0.05$). The frequency of $CXCR2^+$ neutrophils in circulation was mildly increased in aged mice in both treatment groups at days 3 and 7 compared to young animals (**Figure 21D-E**, $p<0.05$). Again, G-CSF treatment did not impact the age-dependent difference in the frequency of $CXCR2^+$ neutrophils. Similar to our previous study, aged animals exhibited elevated expression of CXCR2 at day 3 compared to young animals (**Figure 21F**, $p<0.05$), and G-CSF did not impact the MFI of CXCR2 in either young or aged mice. No age- or G-CSF-dependent differences in CXCR2 expression on circulating neutrophils were observed at day 7 (**Figure 21G**).


A
SSC-A
FSC-A
<PE-Cy7-A>: F480
<FITC-A>: GR1
<PE-A>: CXCR2
<FITC-A>: GR1
Day 3
Day 7
B
C
Young
Aged
Neutrophil Frequency in Peripheral Blood (%)
Frequency of Neutrophils in Peripheral Blood (%)
Saline
G-CSF
D
E
Frequency of CXCR2+ Neutrophils (%)

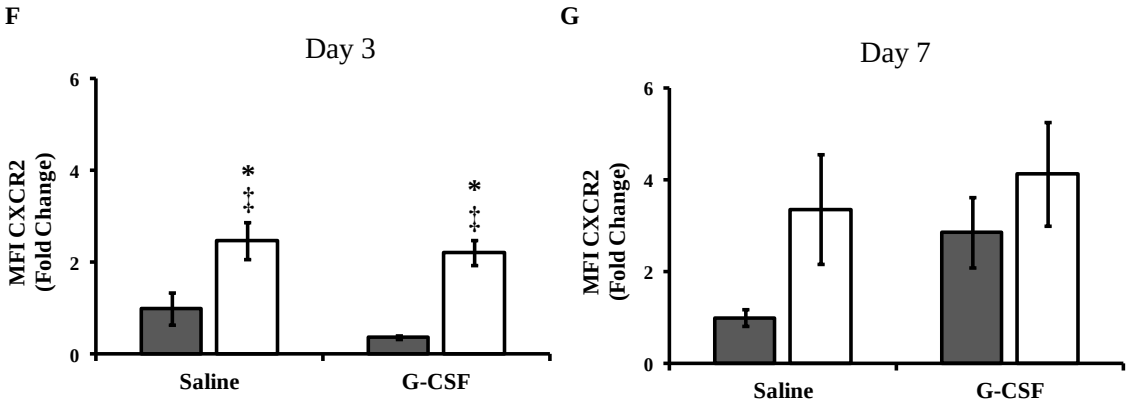


Figure 21. Local G-CSF does not alter the circulating neutrophil pool in aged mice. Whole blood was analyzed by flow cytometry to determine the percentage of circulating neutrophils and expression of the chemotatic receptor CXCR2. (**A**) Gating strategy for neutrophils ($F4/80^{-}Gr\text{-}1^{+}$) in whole blood by flow cytometry. Percentage of circulating neutrophils in peripheral blood in young (gray bars) and aged (white bars) mice at day 3 (**B**) and day 7 (**C**). Frequency of $CXCR2^{+}$ neutrophils in peripheral blood at day 3 (**D**) and day 7 (**E**). Expression of CXCR2 on peripheral blood neutrophils at day 3 (**F**) and day 7 (**G**). Data are shown as mean ± SEM, *$p<0.05$ compared to young saline, ‡$p<0.05$ compared to young G-CSF and §$p<0.05$ compared to young with the same treatment by one-way ANOVA; N=7-14 per group.

Summary

Herein, we have demonstrated that one administration of *s.c.* G-CSF 30 minutes following cutaneous wound infection with *S. aureus* promotes bacterial clearance in an age-dependent manner. The observed reduction in wound bacterial burden at day 3 and 7 in G-CSF-treated aged mice was associated with increased neutrophil accumulation. Other laboratories have shown that G-CSF treatment can enhance age-related deficits in neutrophil chemotaxis. Previously, we have reported that neutrophils from aged mice demonstrate hyperchemokinesis but impaired directional motility *in vitro*. *In vivo* studies confirmed that neutrophils from aged mice have a reduced migratory response toward *s.c.* KC (Brubaker *et. al* 2012, manuscript submitted). We hypothesize that G-CSF restores these chemotatic defects in aged mice, enhancing neutrophil recruitment and helping reduce infection at the wound site in aged mice.

Furthermore, G-CSF enhanced wound closure in aged animals as compared to young. Reports in aged mice indicate that *i.p.* G-CSF administration augments neutrophil recruitment and promotes wound closure in aseptic models of wound healing (49). The necessity of neutrophils for improved wound healing in these studies was confirmed by direct application of peritoneal derived neutrophils to the wound site. Together, these data suggest that neutrophils are required for wound closure in aged mice, and, in the setting of wound infection, increasing neutrophil recruitment promotes resolution of wound infection in aged mice.

We did not observe any systemic effects of G-CSF on the peripheral blood pool. Local, one-dose administration of G-CSF did not significantly enhance the frequency of circulating neutrophils or the expression of their primary chemotatic receptor CXCR2. As

the effect of G-CSF appears to be localized to the wound site in our studies, these data support the potential use of local G-CSF as therapeutic intervention with limited systemic side effects. Future work to elucidate the mechanism by which G-CSF exerts these age-dependent effects will develop our understanding of the aging innate immune system and may reveal therapeutic potential for G-CSF in the wound care setting.

CHAPTER 5

AGE DOES NOT IMPACT ANTIMICROBIAL PEPTIDE EXPRESSION IN A MODEL OF CUTANEOUS WOUND INFECTION

Abstract

Antimicrobial peptides are small cationic molecules that are able to promote bacterial killing via interactions with the bacterial membrane, leading to membrane destabilization and bacterial lysis. In murine studies, mouse β-defensin-3 (mBD3), mBD14, cathelicidin related antimicrobial peptide (CRAMP) and Reg3γ have been shown to possess bactericidal activity against *S. aureus*. Considering the growing interest in these molecules as potential therapeutic targets in the setting of infection, we evaluated the impact of advanced age on cutaneous AMP expression following *S. aureus* wound infection in young (3-4 month) and aged (18-20 month) BALB/c mice. At days 1-10 after injury and infection, mice were sacrificed and AMP expression was evaluated by RT-PCR and immunofluorescence. Age-dependent differences in mRNA levels of mBD3, mBD14 and CRAMP were variable between studies at day 1, with no age-related difference being observed at later time points. In three replicate studies, Reg3γ was suppressed in aged animals as compared to young following injury and infection at day 1. By immunofluorescence, levels of mBD3 expression were low in uninjured skin in young and aged mice. After injury and infection at day 1, young animals had a marked increase in mBD3 expression along the wound edge. Aged animals failed to upregulate mBD3

protein levels as seen by immunofluorescence. TLR2 is a known regulator of AMP expression and the frequency of $TLR2^+$ cells in aged mice was reduced as compared to young. There were no observed differences in the frequency of $TLR2^+$ keratinocytes following injury, or in the MFI of TLR2. These data suggest that age-related differences in the AMP response are variable in our model of cutaneous *S. aureus* wound infection. In particular, levels of mBD3 and mBD14 mRNA are contradictory between studies. While Reg3γ seems to be consistently decreased at the mRNA level in aged mice, further studies to validate these differences at the protein levels should be considered.

Introduction

The failure to effectively heal wounds is estimated to account for over 25 billion in US health care costs, and is augmented by the increasing prevalence of diabetes and obesity (151). The growing elderly population is a major contributor to the elevated prevalence of these chronic conditions, and they are also a major patient population afflicted with chronic wounds (119, 151, 159). Moreover, aging is associated with a precipitous decline in immune function that further compounds the delayed wound closure and heightened risk of infectious complications in the elderly (64, 160, 161). In healthy, non-aged patients, *S. aureus* infection accounts for 30% of surgical site infections as compared to 50% in aged patients, suggesting a penchant of this Gram-positive bacterium for the immunocompromised (159). Antimicrobial peptides, such as defensins and cathelicidins, are small cationic molecules that are able to promote bacterial killing via interactions with the bacterial membrane, leading to membrane

destabilization and bacterial lysis (13). Previously, mouse b-defensin-3 (mBD3), the homolog to human β-defensin 2 (hBD2), and mBD14, the ortholog to hBD3, have been shown to be induced following injury or infection and to possess bactericidal activity against *S. aureus* (15, 16, 134, 137, 201). Cathelicidins, like defensins, have also been show to play critical roles in wound repair and infection. Namely, loss of CRAMP is associated with increased susceptibility to infection and delayed wound closure in murine models. More recently, the Reg family of AMPs are emerging as another important antimicrobial family at the level of the skin and lung. Within this AMP family, Reg3γ is an AMP that is regulated by IL-22, and is most commonly discussed in the context of gut homeostasis and bacterial clearance (202, 203). While few publications discuss the role of the Reg family in skin or any tissue other than the gut, IL-22 has been shown to be a player in skin inflammation [reviewed in (204)]. Recent data has also implicated Reg3γ in methicillin resistant *S. aureus* (MRSA) pneumonia. Despite the knowledge that these AMPs are important in wound healing and resolution of infection, to our knowledge the impact of aging on cutaneous AMPs has not been examined.

As a gram-positive organism, the host response to *S. aureus* is driven in part by recognition via the TLR2 pathway in epidermal keratinocytes and innate immune cells (16, 98, 181, 205-207). Increased susceptibility to *S. aureus* infection is related to loss of TLR2 (98) by affecting neutrophil recruitment to the infection site (181) and through decreased expression of cutaneous AMPs in response to infectious challenge (16, 208). In addition to evaluating AMP expression, we sought to determine if differences in

keratinocyte TLR2 expression could contribute to potential age-related differences in antimicrobial peptide levels.

We examined AMP levels in young and aged mice following cutaneous wound infection with *S. aureus*. Levels of mBD3, mBD14 and CRAMP yielded inconclusive results concerning an age-dependent change in these molecules. However, Reg3γ was consistently suppressed in aged mice after injury and infection as compared to young. These data suggest that Reg3γ levels may be reduced with aging; however, further studies should be conducted to more conclusively evaluate the impact of age on AMP expression following injury and infection.

Materials and Methods

Animal Model

3-4 month old (young) and 18-20 month old (aged) BALB/c mice (Charles River/NIA, Kingston Facility, Stony Ridge, NY) were utilized to determine age-dependent differences in response to cutaneous wound injury and infection (**Figure 5**). All animal studies were approved and performed with strict accordance to the regulations established by the Loyola University Chicago Animal Care and Use Committee. Following acclimation at Loyola's Animal Care Facility, young and aged mice were subjected to dorsal excisional cutaneous injury as previously described (175). Briefly, mice were administered 100 mg/kg ketamine and 10 mg/kg xylazine *i.p.* followed by *i.p*. saline to ensure systemic distribution of the anesthetic. Once the mice no longer responded to firm pressure applied to their hind limb, their dorsum's were shaved and cleansed with ethanol

pads. Mice were then subjected to 6 dorsal full-thickness (skin and panniculus carnosa) cutaneous wounds with a 3 mm dermal punch biopsy (Acuderm, Ft. Lauderdale, FL). Immediately after injury, mice received ~10^3 CFU/10uL of *S. aureus* and were returned to their cages on heating pads. A low inoculum of bacteria was chosen to prevent sepsis which is known to negatively impact wound healing (176). *S. aureus* Newman strain was grown overnight in TSB at 37^{o}C under constant agitation. The next day, 1 mL of *S. aureus* in TSB was resuspended in 2mL fresh TSB and incubated at 37^{o}C for 2 hours to ensure mid-logarithmic growth at the time of application to cutaneous wounds. Bacterial concentration (CFU/mL) was determined by absorbance at 600 nm and the final inoculum confirmed by back-plating on MSA (BD Diagnostics, Sparks, MD).

Mice were sacrificed at days 1, 3, 7 and 10 after injury and infection. Wounds were excised and examined for AMPs via RT-PCR and immunofluorescence as described below. Two additional wounds, or two 5 mm punches of uninjured skin, were used to evaluate keratinocyte expression of TLR2.

RT-PCR

Following sacrifice, one wound was excised with a 5 mm punch biopsy and RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was quantified using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE) and cDNA synthesized using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA). Quantitative RT-PCR was performed with the Applied Biosystems 7500 Fast Real-Time PCR system, using specific TaqMan Expression Assays (Applied

Biosystems) for mBD3, mBD14, CRAMP, Reg3γ, K14 and β-actin. Values were then normalized to β-actin expression via the ΔΔCT method. For re-expression to K-14, each data set was normalized to β-actin, and then expressed as the normalized gene of interest: normalized K14.

Immunofluorescence

Following sacrifice, a 5mm punch biopsy was used to removed the injured tissue and tissue borders. The skin was frozen in OCT, sectioned at 0.7 microns and frozen. At the time of analysis, slides were thawed overnight at room temperature in a humidified chamber and then fixed in 4% paraformaldehyde for 15 minutes. Slides were washed 3 times with PBS, blocked with 10% BSA with 3% normal goat serum in PBS for 1 hour and stained overnight at 4^{o}C with rabbit anti-human mBD2 with known cross-reactivity to mBD3. The following day, slides were washed 3 times with PBS and stained with goat anti-rabbit-Alexa-488 for 1 hour. Slides were washed three times in PBS and coversliped using VECTASHEILD mounting media with DAPI for nuclear staining (Vector Laboratories, Burlingame, CA). Fluorescent images were captured using a Zeiss Axiovert 200 microscope (Zeiss, Thornwood, NY).

Skin Cell Isolation and Flow Cytometry

Single cell suspensions of wound cells for flow cytometry were generated as previously described (177). At days 1-10 after injury and infection, animals were euthanized, pelts removed and wounds excised using a 5 mm punch biopsy. Two diced

wounds were incubated overnight at 4^{o}C in RPMI 1640 culture media (Gibco, Grand Island, NY) containing 10% FBS (Hyclone, Logan, UT), 2 mM L-glutamine (Gibco, Grand Island, NY), 1% penicillin/streptomycin (Gibco, Grand Island, NY), 2 mg gentamycin sulfate (Mediatech Inc, Manassas, VA) and 0.3 mg dispase II (Roche Diagnostics, Indianapolis, IN). Tissue pieces were then removed and subjected to further enzymatic digestion with 1 mg collagenase from *Clostridium histolyticum* type 1A (Sigma-Aldrich, St. Louis, MO), 1.2 mg DNAse I from bovine pancreas Grade II (Roche Diagnostics, Indianapolis, IN), 1 mg hyaluronidase from bovine testes type 1-S (Sigma-Aldrich, St. Louis, MO), in RPMI 1640 with 5% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, gentamycin and magnesium chloride hexahydtrate for 2 hours at 37^{o}C. After two hours, these solutions were combined and adherent cells were treated with Accutase (eBioscience, San Diego, CA) for 8 minutes at 37^{o}C followed by vigorous pipetting. Single cells suspensions were filtered through at 70 μm filter to remove debris. Cells were washed and adjusted to 1x10^{6}/mL. Wound cells were blocked for 20 minutes with FcBlock (anti-CD16/CD32, eBioscience) and rat IgG (Jackson ImmunoResearch, West Grove, PA), and then stained with APC-EpCAM (eBioscience, San Diego, CA), and PE-TLR2 (eBioscience, San Diego, CA). Cells were washed twice and then resuspended in flow buffer (1% BSA (Sigma-Aldrich, St. Louis, MO), 0.1% sodium azide and 2mM EDTA in PBS). Events were collected on the FACS LSR Fortessa (BD Biosciences, San Jose, CA) and data were analyzed by FlowJo Software (Tree Star Inc, Ashland, OR). Fluorescence minus one control staining and single color controls were used to determine positive staining. EpCAM^{+} cells were considered keratinocytes.

Statistical analysis

Data are shown as mean ± SEM of each group. Data were analyzed by Student's *t* test or one- way or two-way ANOVA with Tukey's or Bonferroni post-hoc tests, respectively, where appropriate using GraphPad Prism 5 (GraphPad, La Jolla, CA). A value of $p \leq 0.05$ was considered significant.

Results

mRNA levels of cutaneous antimicrobial peptides: mBD3, mBD14 and CRAMP

To evaluate the impact of advanced age on AMP expression following cutaneous injury and infection, we examined mRNA levels of mBD3, mBD14, CRAMP and Reg3γ. Data from our first experiments suggested a lack of induction of mBD3 and 14 at day 1 following injury and infection in aged mice as compared to young, with no age-related differences noted in mRNA levels of CRAMP (**Figure 22A-C).** At days 3 and 7 after injury and infection, there is a trend towards elevated mBD3 mRNA levels in aged mice as compared to young in two replicate experiments, but these data were not significant. No consistent difference between age groups were observed for mBD14 at these later time points (**Figure 22D-E**).

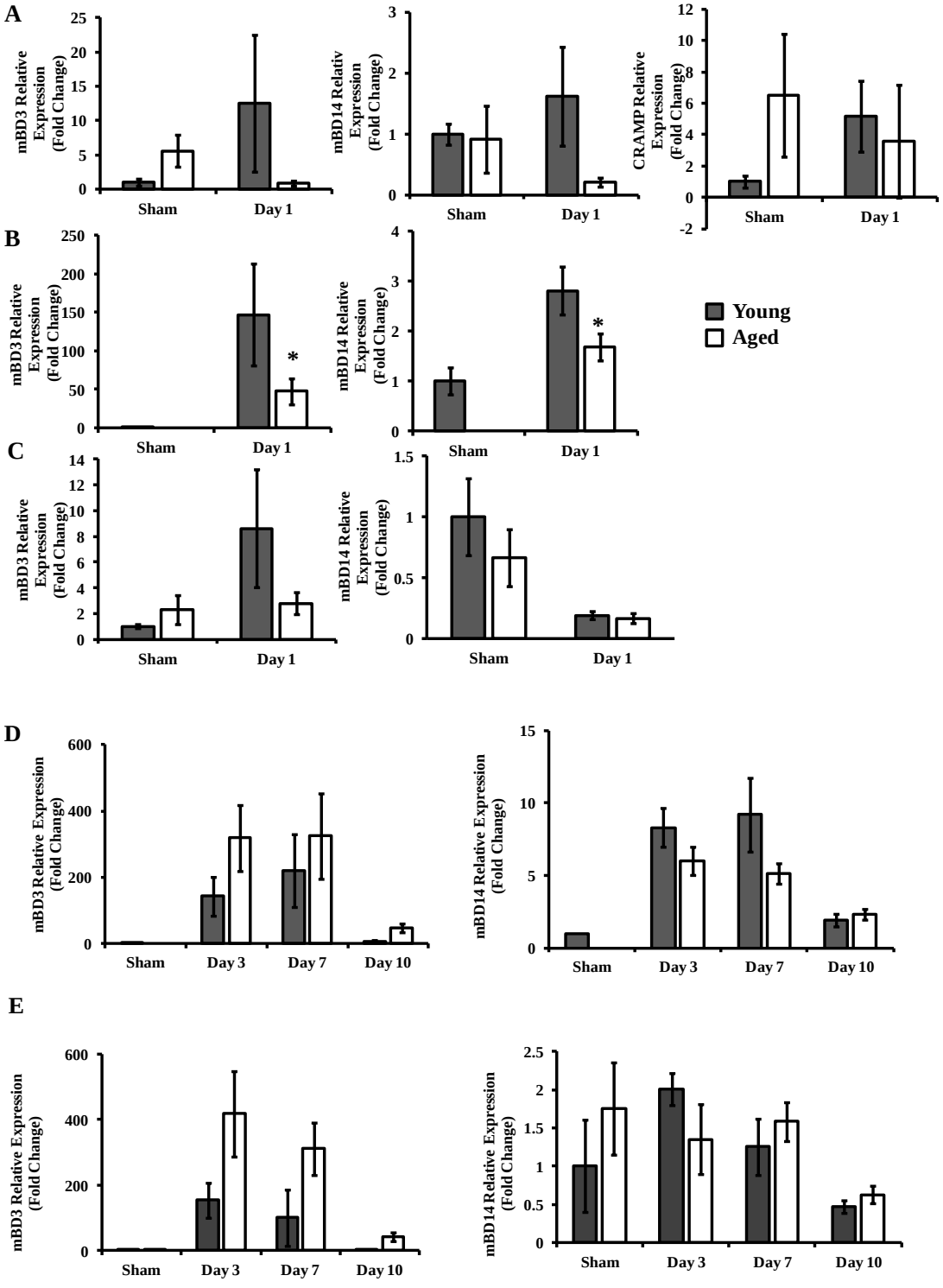

A
mBD3 Relative Expression (Fold Change)
mBD14 Relativ Expression (Fold Change)
CRAMP Relative Expression (Fold Change)
Sham
Day 1
B
mBD3 Relative Expression (Fold Change)
mBD14 Relative Expression (Fold Change)
*
Young
Aged
C
mBD3 Relative Expression (Fold Change)
mBD14 Relative Expression (Fold Change)
D
mBD3 Relative Expression (Fold Change)
mBD14 Relative Expression (Fold Change)
Sham
Day 3
Day 7
Day 10
E
mBD3 Relative Expression (Fold Change)
mBD14 Relative Expression (Fold Change)

Figure 22. Cutaneous antimicrobial peptide expression in young and aged mice following cutaneous *S. aureus* infection. Skin was isolated from young (gray bars) and aged (white bars) mice pre- and post-injury and infection. RNA was isolated from wound homogenates and gene expression of mBD3, mBD14 and CRAMP were determined by RT-PCR. (**A-C**) Relative gene expression of mBD3, mBD14 and/or CRAMP from three independent experiments in uninjured skin and at day 1 following cutaneous wound infection. (**D-E**) Relative gene expression of mBD3 and mBD14 at days 3, 7 and 10 after injury and infection from two independent experiments. Data are expressed as fold change compared to young sham and shown as mean ± STDEV (**A**) or SEM (**B-E**); *$p<0.05$ by Student's t-test. (**A**) N=2-3 per group; (**B**) N=3-5 per group; (**C**) N=3-4 per group; (**D-E**) N=3-6 per group.

These data suggested a lack of induction of mBD3 and mBD14 at day 1 following injury and infection in aged mice as compared to young, suggesting a plausible mechanism for the elevated bacterial colonization and delayed leukocyte recruitment observed in aged animals. To determine if differences at the mRNA levels translated into differences at the protein level mBD3 protein expression at day 1 post injury and infection by immunofluorescence (**Figure 23**). In intact, uninjured skin obtained from young and aged sham mice, the levels of mBD3 were very low (**Figure 23A-B**). At day 1 after cutaneous wound infection, aged mice demonstrated a lack of induction of mBD3 along the wound border as compared to young mice (**Figure 23C-D**). These images are representative of 5 animals per group from the experiment shown in Figure 22B.

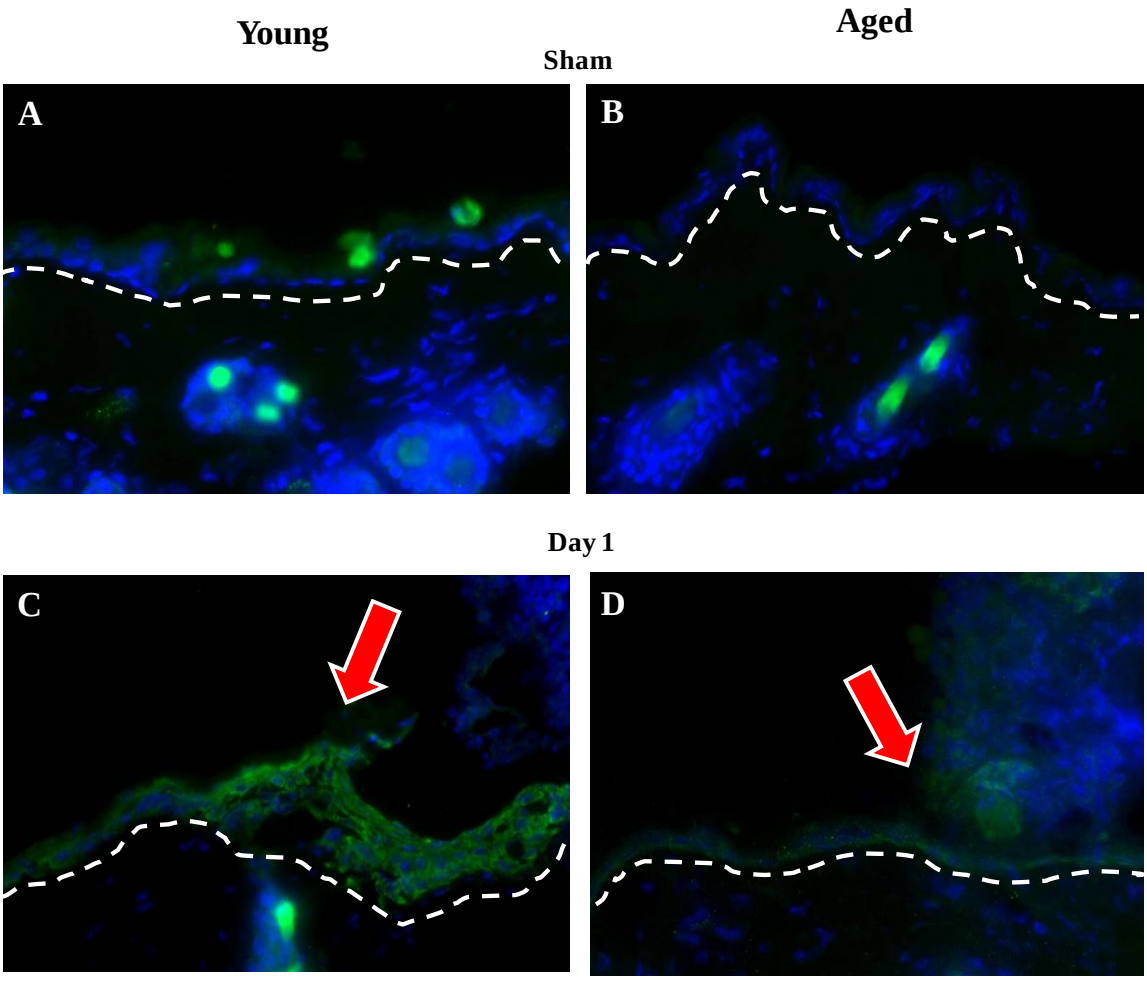


Figure 23. Fluorescent microscopy of mBD3 expression following cutaneous wound infection in young and aged mice. Uninjured skin and cutaneous wounds excised at day 1 after injury were sectioned, fixed, blocked and then stained overnight at 4^{o}C with rabbit anti-hBD2. Slides were then incubated with goat anti-rabbit AF488 (green) and DAPI (blue). Young sham (**A**), aged sham (**B**), young wound infection (**C**) and aged wound infection (**D**). Red arrows indicate the wound margin. Note: intense accumulation of green dye in a circular/oval shape in **A-C** is associated with hair follicles. Fluorescent images were acquired on a Zeiss Axiovert 200. Images representative of N=5 animals per group from the experiment shown in Figure 22B.

As the relative level of mRNA expression was variable between experiments, an additional experiment was performed to evaluate mBD3 and mBD14 mRNA levels. Wounds were analyzed by RT-PCR for mBD3 and mBD14, and in contrast to previous experiments, mBD3 expression was elevated in aged mice as compared to young (**Figure 24A**, *****$p<0.05$). No induction of mBD14 was observed, similar to Figure 22C (**Figure 24A**). After consideration of these data, another experiment with larger animal numbers was conducted to evaluate mBD3 and mBD14 at day 1 post injury and infection. These data paralleled the data shown in Figure 24A. At day 1 following cutaneous *S. aureus* infection, aged mice demonstrated elevated expression of mBD3 compared to their younger counter parts (**Figure 24B**, *$p<0.05$). While mBD14 was induced in this study, the relative expression of mBD14 was comparable between young and aged mice (**Figure 24B**). These data are in vast contrast to the first three studies, and have made it difficult to determine the role defensins may play in the age-dependent differences observed in our model.

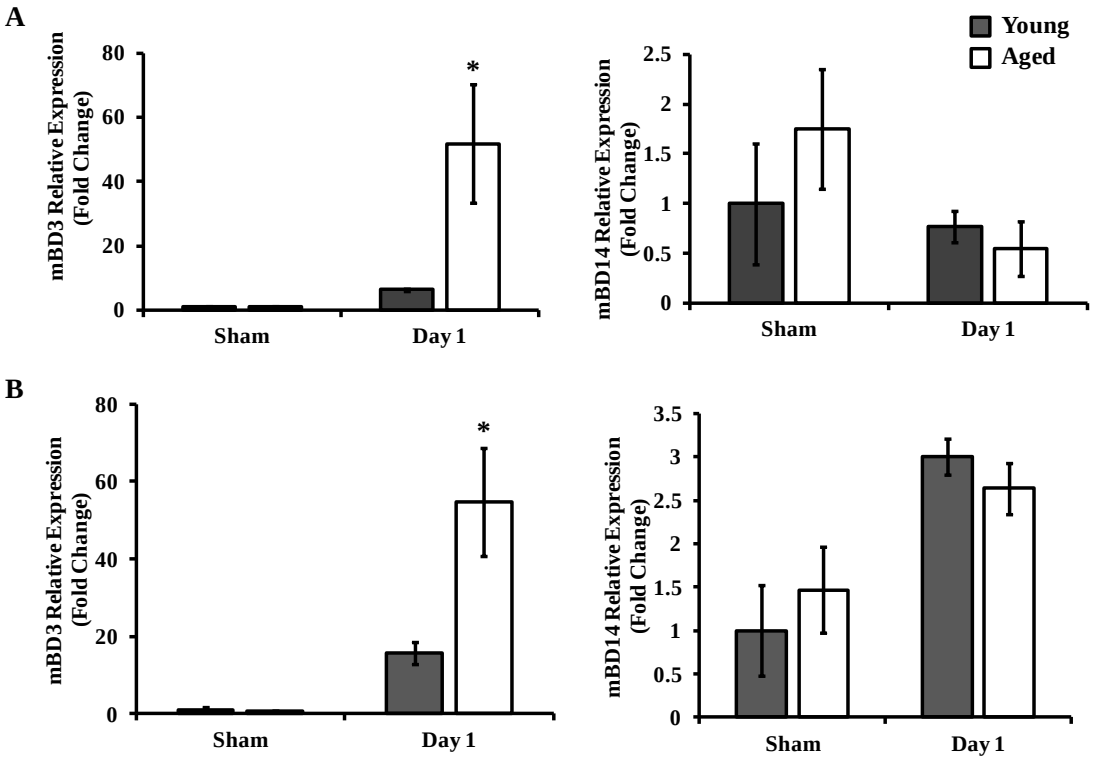


Figure 24. Cutaneous antimicrobial peptide expression in young and aged mice, additional experiments. In two additional replicate experiments, skin was isolated from young (gray bars) and aged (white bars) mice pre- and post-injury and infection. RNA was isolated from wound homogenates and gene expression of mBD3 and mBD14 were determined by RT-PCR (**A-B**). Data are expressed as fold change compared to young sham and shown as mean ± SEM; *$p<0.05$ by Student's t-test. (**A**) N=3-6 per group; (**B**) N=4-10 per group.

In an effort to reconcile these divergent data, and to determine the relative amount of each AMP to keratinocytes in our samples, these data were re-expressed to K14, a keratinocyte marker (**Figure 25**). Re-expression of data to K14 did not significantly alter the trends seen in previous experiments, and suggest to conclusive age-dependent difference in mBD3 or mBD14 levels. To confirm our protein expression previously observed by IF, mBD3 and mBD14 ELISAs were purchased to quantify age-dependent differences. However, these ELISAs failed to develop and similar problems with two subsequent ELISA kits from this company were noted. Taken together our data remain inconclusive regarding the expression of mBD3 and mBD14 after cutaneous injury with advanced age.

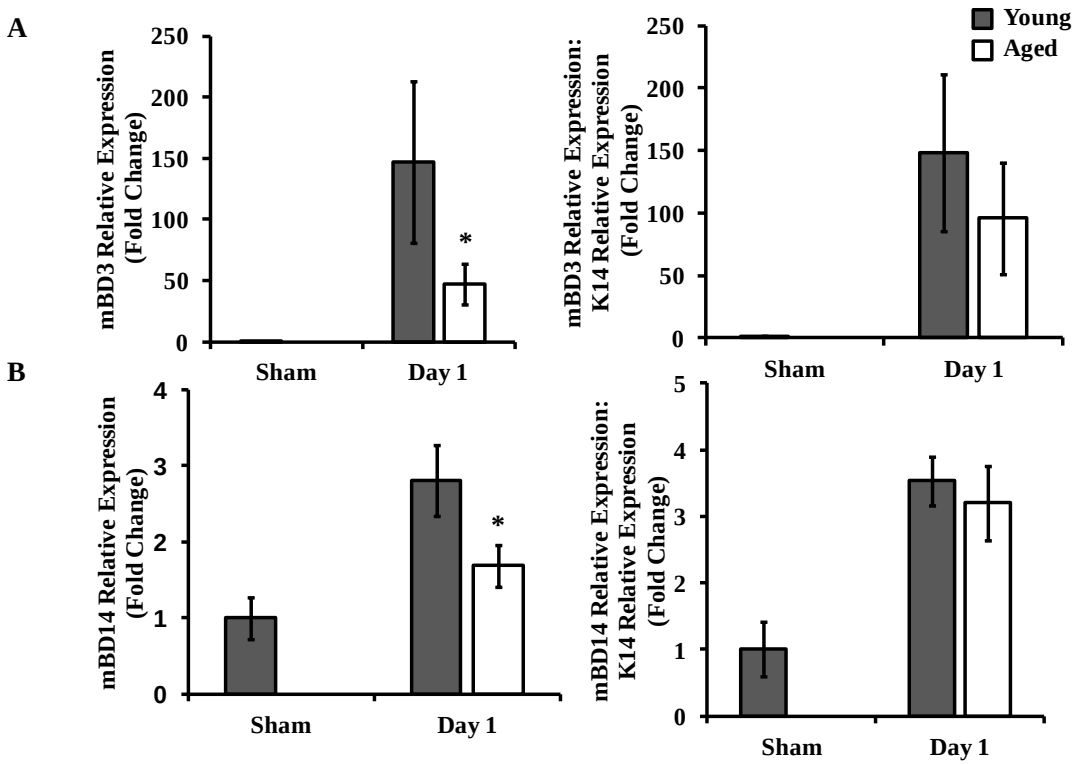


Figure 25. Cutaneous antimicrobial peptide expression relative to K14 in young and aged mice. Skin was isolated from young (gray bars) and aged (white bars) mice pre- and post-injury and infection. (**A**-**B**) Gene expression of mBD3 and mBD14 re-expressed relative to K14 and shown as fold change compared to young sham with mean ± SEM; *$p<0.05$ by student's t-test; N=3-5 per group.

Reg3γ expression following cutaneous S. aureus wound infection

Reg3γ is another AMP regulated by IL-17 and -22, and has been shown to play a role in MRSA infection and tissue injury (REFS). Following cutaneous injury and infection at day 1, we observed a suppression in Reg3γ expression in aged animals relative to young in three replicate experiments (**Figure 26**, $p<0.05$). These differences were maintained after re-expression of the data to K14 (**Figure 27**). While these studies suggest that Reg3γ mRNA levels may be reduced in aged animals compared to young following *S. aureus* wound infection, further work to confirm these studies at the protein level should be conducted.

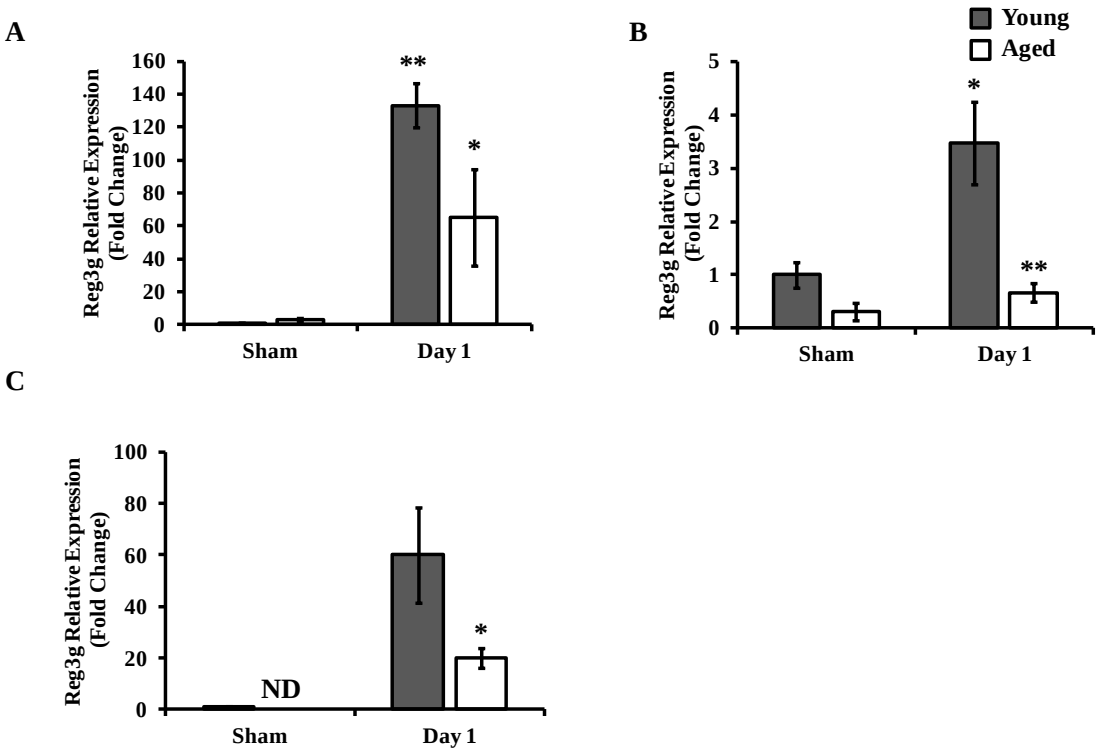


Figure 26. Reg3γ gene expression in young and aged mice. Skin was isolated from young (gray bars) and aged (white bars) mice pre- and post-injury and infection. RNA was isolated from wound homogenates and gene expression of Reg3γ was determined by RT-PCR. Data are expressed as fold change compared to young sham and shown as mean ± SEM; (A) N= 4-10 per group, **p<0.01 versus young sham, *p<0.05 versus young wound+infection by one-way ANOVA; (B) N=4-7 per group, *p<0.05 versus young sham, **p<0.01 versus young wound+infection by one-way ANOVA; (C) *p<0.05 versus young wound+infection by Student's t-test, N= 2-5 per group.

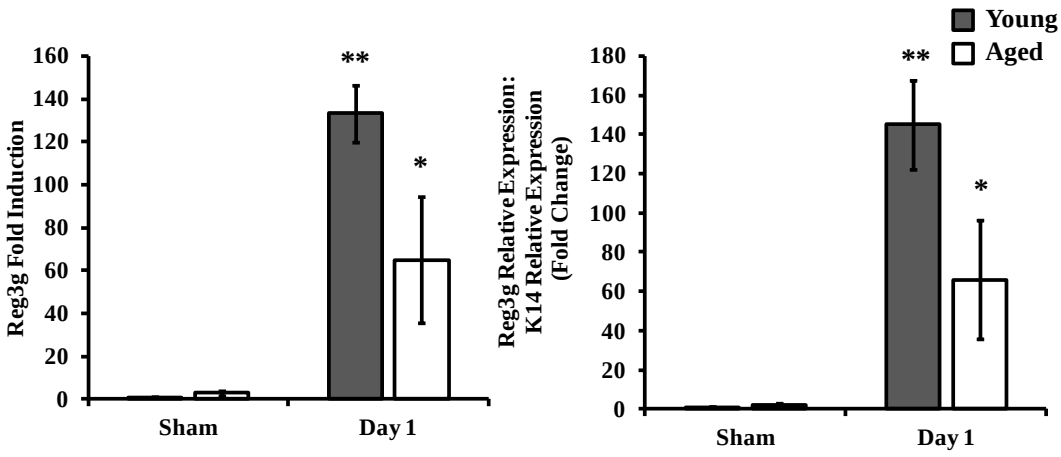


Figure 27. Reg3γ gene expression relative to K14 in young and aged mice. Skin was isolated from young (gray bars) and aged (white bars) mice pre- and post-injury and infection. RNA was isolated from wound homogenates and gene expression of Reg3γ and K14 were determined by RT-PCR. Data are re-expressed relative to K14 and shown as fold change compared to young sham with mean ± SEM. N= 4-10 per group, **$p<0.01$ versus young sham, *$p<0.05$ versus young wound+infection by one-way ANOVA.

TLR2 and CD14 expression on keratinocytes as regulators of AMP expression

TLR2 is the pattern recognition receptor that aids in recognition of the *S. aureus* via homodimerization, or heterodimerization with either TLR1 or TLR6. Binding of *S. aureus* to TLR2 results in recruitment of CD14 and initiation of the MyD88 dependent pathway, resulting in activation of NF-κB and the innate immune response (98, 205). Moreover, TLR2 expression on keratinocytes has been shown to play a role in induction of defensins (16). Expression of TLR2 on keratinocytes was examined by flow cytometry (**Figure 28A**). The epithelial cell population was determined by using the surface marker epithelial cellular adhesion molecule (EpCAM) (209). The frequency of $EpCAM^+$ cells did not differ between uninjured young and aged mice (**Figure 28B**). A reduction in the frequency of $EpCAM^+TLR2^+$ cells found in injured skin from aged mice was observed (**Figure 28C**, $*p<0.05$), though no differences were seen in TLR2 expression between the two age groups prior to injury and infection (**Figure 28D**). Following cutaneous wound infection, the frequency of $EpCAM^+$ cells was decreased in both young and aged animals, however no differences were observed between age groups (**Figure 28E**). Similar to uninjured young and aged mice, no age-associated differences in the frequency of $EpCAM^+TLR2^+$ cells or the MFI of TLR2 on $EpCAM^+TLR2^+$ cells were observed (**Figure 28F-G**).

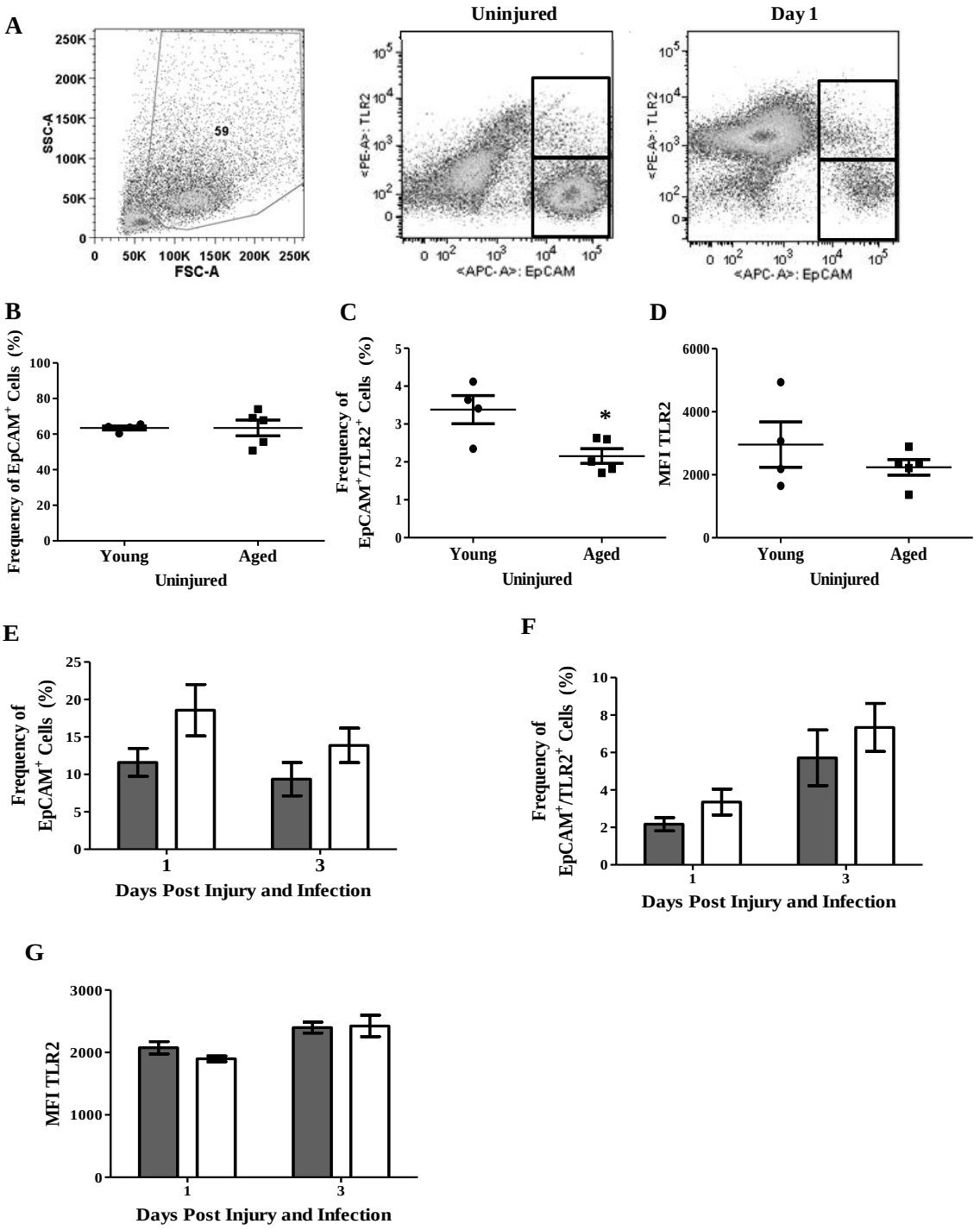


Figure 28. Keratinocyte expression of TLR2 pre- and post-cutaneous wound infection. Expression of TLR2 was determined by flow cytometry with $EpCAM^+$ cells being considered the keratinocyte and epithelial cell population (**A**). (**B**) Frequency of

$EpCAM^+$ cells in young (circles) and aged (squares) mice prior to injury and infection. (**C**) Frequency of $EpCAM^+TLR2^+$ cells in young and aged mice in uninjured skin. (**D**) MFI of TLR2 on $EpCAM^+TLR2^+$ cells prior to injury and infection. (**E**) Frequency of $EpCAM^+$ cells at days 1 and 3 after injury and infection. (**F**) Frequency of $EpCAM^+TLR2^+$ cells following cutaneous wound infection at days 1 and 3. (**G**) MFI of TLR on $EpCAM^+TLR2^+$ cells after injury and infection. Data are shown as mean ± SEM, N=4-6 per group,*$p<0.02$.

No differences in the frequency of $EpCAM^{+}TLR2^{+}CD14^{+}$ cells were noted between young and aged mice prior to injury or at day 1 post injury (**Figure 29A+C**). The MFI of CD14 on $TLR2^{+}$ keratinocytes was similar between young and aged uninjured mice, however an increase in the MFI of CD14 was seen in aged animals compared to young at 1 day after injury and infection (**Figure 29B+D**, $p<0.05$). These data suggest that age-depended difference in TLR2 levels in uninjured keratinocytes could contributed to increased susceptibility to Gram-positive infection with advanced age; however, differences in TLR2 or CD14 on keratinocytes after injury and infection do not play a role in altered resolution of wound infection in aged mice.

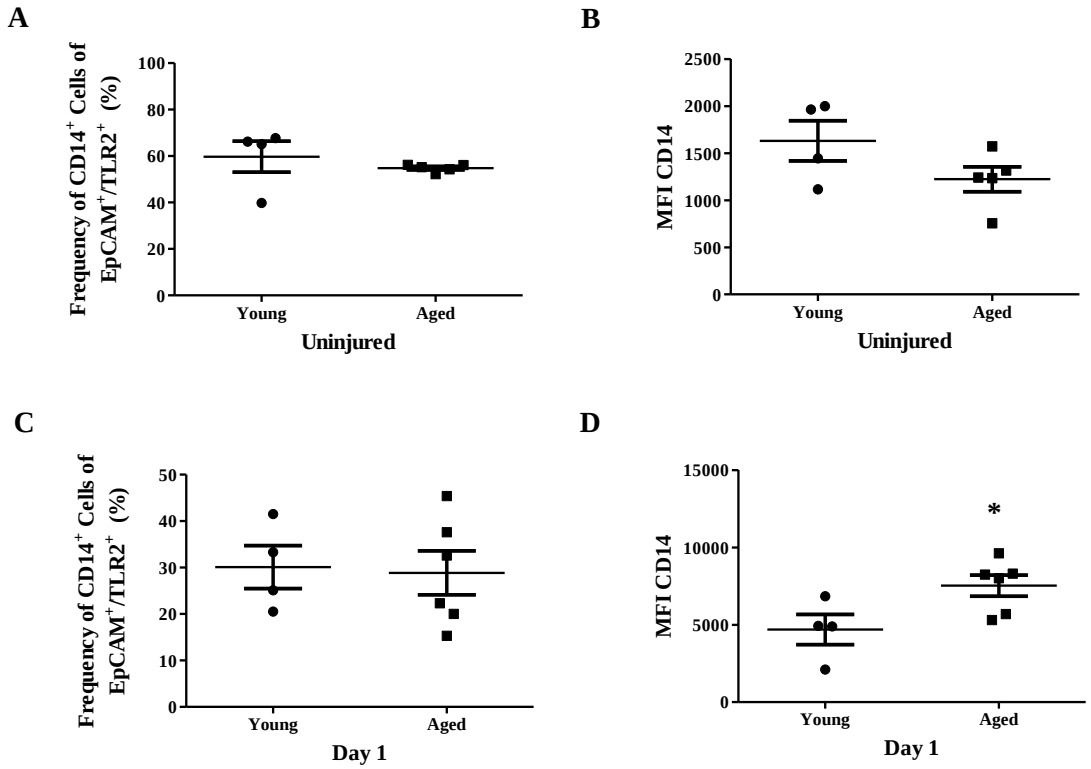


Figure 29. Expression of CD14 on TLR2$^+$EpCAM$^+$ cells pre- and post-cutaneous wound infection. Expression of CD14 and TLR 2 were determined by flow cytometry with EpCAM$^+$ cells being considered the keratinocyte and epithelial cell population. (**A**) Frequency of CD14$^+$ cells within the EpCAM$^+$TLR2$^+$ population in young (circles) and aged (squares) mice prior to injury and infection. (**B**) MFI of CD14 on EpCAM$^+$TLR2$^+$CD14$^+$ cells in uninjured skin. (**C**) Frequency of CD14$^+$ cells within the EpCAM$^+$TLR2$^+$ population at day 1 after injury and infection. (**B**) MFI of CD14 on EpCAM$^+$TLR2$^+$CD14$^+$ cells at day 1 after injury and infection. Data are shown as mean ± SEM, N=4-6 per group,*$p<0.02$.

Summary

Following *S. aureus* wound infection we observed varying degrees of induction of mBD3, mBD14 and CRAMP in young and aged animals. Interestingly, it appears that peak levels of these AMPs occurred between days 3 and 7. Generally, AMPs are considered early mediators of the innate immune response to cutaneous wound injury; however, the infection may be generating a protracted AMP response in our system. As we did not observe consistent differences in mBD3, mBD14 or CRAMP at the mRNA level, we cannot conclude the impact of age on these mediators. To our knowledge, the only published study that examined the effect of age on AMP expression examined levels of hBD2, the human homolog to mBD3, in uninjured skin from young and elderly subjects. In this study, basal levels of hBD2 were reduced in uninjured skin from elderly subjects as compared to young. We did not observe this same trend in uninjured murine skin. In regards to TLR2 expression, various TLRs on differing cell types have been showed to be affected by age. We did observe a reduction in the frequency of $TLR2^{+}$ cells with age prior to injury, but following injury and infection these differences were no longer observed. As TLR2 is important in the immediate host response to Gram-positive infection, this basal decrease in TLR2 expression with age may enhance susceptibility to infection associated with aging. Future work to evaluate TLR1/6 expression, the two co-receptors for TLR2, in aging skin would strengthen our knowledge regarding cutaneous TLRs and their role in vulnerability to *S. aureus* infection with age. Additional work to evaluate how aging alters AMPs following injury and infection should also be examined.

CHAPTER 6

DISCUSSION

In review, we have developed a murine model of cutaneous wound infection with *S. aureus* that allows for evaluation of age-dependent differences in the immune response to infection and tissue injury. Herein, we demonstrated that advanced aged is associated with elevated bacterial colonization at early time points, and persistent infection out to day 10 post wound infection. These findings parallel clinical observations in elderly patients as they have elevated rates of *S. aureus* infection, which correlates with a prolonged length of hospital stay and increased hospital costs (121, 159, 174). Other murine models of infection, such as pneumonia, also report an increased bacterial burden in aged animals (192). Moreover, we observed that wound closure was protracted in aged animals. Studies in murine models of uninfected wounds report similar delays in wound closure with advanced age (49, 63); however, the time to closure was reduced as compared to infected wounds from our animals. Interestingly, despite the persistent elevation in bacterial burden observed in the aged mice, the delay in wound closure was not perturbed to a similar degree. This may be due to the relatively low inoculum used in this study to limit septic events known to alter wound healing (176). Alternatively, the persistence of bacteria in the wound bed may be altering other components of wound repair such as collagen deposition which aids in restoration of the dermal matrix (63, 210). While not evaluated in this study, further work examining how bacterial infection

alters the wound collagen matrix and tensile strength may help elucidate mechanisms underlying the elevated rate of wound dehiscence in elderly patients.

An important factor in mediating the host response to infection is early recognition of foreign pathogens by host immune cells. Following tissue injury, local and recruited leukocytes act to detect evolutionary conserved PAMPs via pattern recognition receptors (PRRs). TLR2 is a PRR which specifically recognizes Gram-positive bacteria via homodimers or heterodimers with TLR1 and TLR6 (211). Activation of TLRs in innate immune leukocytes results in NF-kB dependent production of pro-inflammatory mediators, helping to recruit additional leukocytes and promote antigen presentation to recruited adaptive immune cells (100, 182). Loss of TLR2, or mediators of TLR signaling, have been associated with increased susceptibility to *S. aureus* infection, as mice deficient in TLR2 or MyD88 demonstrated heightened levels of *S. aureus* bacteremia (98, 181, 183, 212). Thus, we chose to evaluate TLR2 expression on keratinocytes as well as resident and infiltrating leukocytes, as TLR2 is required to effectively eliminate *S. aureus*. In uninjured skin, aged mice had a reduced frequency of $TLR2^+$ keratinocytes relative to young mice, though no difference in the MFI were noted. Similarly, we observed an age-dependent decrease in the absolute number of $TLR2^+$ resident tissue macrophages; however, no differences in expression or frequency of $TLR2^+$ infiltrating wound neutrophils or macrophages were observed. Together, the reduction in the frequency of $TLR2^+$ keratinocytes and resident macrophages may contribute to heightened vulnerability to infection with advanced age. Other aging studies have found variable results in regards to tissue macrophage TLR2 expression (74). While reports demonstrate that macrophage expression of one or all TLRs are reduced with

aging (74, 101, 192), previous studies from our own laboratory have demonstrated no change in peritoneal or splenic macrophage TLR2 and TLR4 expression (82). In circulating neutrophil populations, human studies suggest that expression of TLR2 and TLR4 are unaltered with age, though membrane-associated MyD88 was decreased with age after exogenous stimulation with LPS (45). Though not evaluated in our study, age-associated alterations in mediators of TLR2 signaling, such as MyD88, Toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) or interleukin-1 receptor-associated kinase (IRAK), may dampen TLR2 responses in aged individuals and contribute to delayed resolution of bacterial infection (100, 213, 214).

TLRs are also known to initiate the signaling cascade that results in elevated AMP expression following cutaneous injury or infection. Despite reduced TLR expression in aged animals relative to young, we did not observed a basal reduction in AMPs levels with age. This is contrary to a study in humans in which hBD2, the homolog to mBD3, was reduced in skin from elderly subjects as compared to young. While no other studies have evaluated AMP expression following injury and infection in the setting of aging, our contradictory data suggest that further work should be conducted to elucidate if aging impacts these critical early mediators of the innate immune response. Particular attention to be paid to the Reg family, namely Reg3γ, as this appears to be most consistent in our studies and has been shown to be critical in other models of *S. aureus* infection.

In addition to recognition of foreign microbes, neutrophils and macrophages that are recruited to the site of tissue injury play a critical role in phagocytosis and killing of

invading bacteria. Previous studies have reported that aging negatively impacts the ability of both neutrophils and macrophages to phagocytosis pathogens (46, 50, 120, 185, 186), though few have evaluated the phagocytic potential of leukocytes following recruitment in response to tissue injury or infection. Following cutaneous wound injury, no age-dependent differences in wound neutrophil or macrophage phagocytosis were noted. Interestingly, studies in human neutrophils have reported an age-associated reduction in TREM-1 mediated phagocytosis and ROS generation that mediates bactericidal activity in human neutrophils (215). Furthermore, aging was associated with decreased FcγRIII expression in circulating human neutrophils and this correlated with reduced phagocytosis (46). Though our observed differences in FcγRIII did not contribute to differences in phagocytosis or bactericidal activity as reported in human peripheral blood neutrophils by Butcher *et al*., recent reports show that FcγRII regulates FcγRIII with functional effects on phagocytosis (216, 217). Given that we did not find a functional defect in our cell populations, we did not evaluate whether expression of FcγRII, or other phagocytic pathways such as complement-mediated phagocytosis, were altered with advanced age. Moreover, that lack of age-related differences in neutrophil phagocytosis is in concert with other reports in mice that do not recapitulate the phagocytosis deficits observed in circulating human neutrophils (74). Our study extends these findings in rodent studies to demonstrate that even after recruitment to a specific tissue microenvironment, neutrophil phagocytosis is not impaired by advanced age.

On the other hand, human and rodent studies more definitively agree that aging impairs macrophage phagocytosis (50, 185-187, 218), though some reports find no age-

dependent phagocytic differences (74, 83). DiPietro *et al.* examined wound macrophage phagocytosis in young and aged mice with divergent results from those observed in this study. Following isolation of macrophages from subcutaneous implanted PVA sponges, phagocytosis was reduced ~40% in macrophages from aged mice relative to young mice (50). While this study also examined the phagocytic potential of cells following recruitment to cutaneous tissue, it did so by implementing PVA sponges to isolate recruited macrophages (219). This environment is different than that created by the wound milieu of an excisional wound, and may account for the observed differences. It is also important to note that the wound cell isolation procedure and optimal conditions of the pHrodo-*S. aureus* phagocytosis assay may limit observing small age-dependent impairments of phagocytosis in either wound neutrophils or macrophages.

Most significantly, our data supports the idea that advanced age alters the infiltration kinetics of innate immune cells following tissue injury and infection. Alterations in infiltration of these cells can impact several facets of wound healing. First, reduced recruitment of these phagocytes may directly contribute to heightened bacterial levels as these infiltrating phagocytes are critical in controlling infectious spread (193). Second, the age-dependent differences in the initial wave of neutrophil infiltration may affect recruitment of macrophages via secreted mediators that directly interact with macrophage chemokine receptors and the vascular endothelium (172, 220). A delay or reduction in macrophage accumulation, or alteration in macrophage phenotype, may further impair transition to the proliferative phase of wound healing, negatively impacting wound closure and restoration of the dermal matrix (118, 144, 221). Moreover, diminished macrophage function and/or numbers have been shown to delay wound

healing and decrease wound tensile strength (145, 147). Lastly, studies in aged mice suggest that there is a necessity for neutrophils in efficient wound healing with advanced age (26). However, these data call into question the accepted dogma of efficient wound healing. While macrophages are considered to be beneficial to wound healing, the role of neutrophils in sterile wound healing is controversial. Although neutrophils are required for eradication of invading organisms, gratuitous neutrophil accumulation can result in excessive ROS and elastase production, in turn exacerbating tissue damage and impeding wound healing (65, 160). Specifically, in fetal models of wound healing, there is a paucity of neutrophil recruitment and inflammation that is correlated with reduced scar formation (131, 222). Studies in young mice have also shown that neutrophil depletion may accelerate aseptic wound closure (140). Despite these findings, reports in the aging literature have demonstrated that even in the setting of sterile wound healing, ablation of the neutrophil population delays wound closure in aged animals (49). Importantly, increasing neutrophil numbers via *i.p.* administration of G-CSF enhanced rates of wound closure in aged mice to those observed in young mice (49). In the context of an infected wound, the necessity of neutrophil recruitment becomes increasingly paramount. Clinically, patients with reduced neutrophil function or numbers are at a heightened risk for chronic and repeated infections with catalase-positive bacteria, like *S. aureus* (43, 184, 194). Thus, the decreased absolute numbers of neutrophils and macrophages in our model may not only contribute to bacterial colonization differences observed with age, but may also play a role in delayed wound resolution in aged animals.

As mentioned previously, this weakened neutrophil response may alter the kinetics and subsequent leukocyte infiltration of wound healing in aged animals.

Recently, we reported that peripheral blood neutrophils from unmanipulated young and aged mice have a basal hyperchemokinesis but lack directional migration *ex vivo* (60). Other studies have supported that aging is correlated with neutrophil chemotatic defects (45, 74, 200). Here, we extend these findings to show that at increasing doses of KC *in vivo*, neutrophils from aged mice had a diminished migratory response to cutaneous tissue as compared to young. While we saw blunted chemotaxis to the CXCR2 ligand KC, we observed elevated CXCR2 expression on circulating neutrophils from aged animals. These data suggest that despite adequate expression of CXCR2, mediators of CXCR2 signaling may be impaired with age. For example, recruitment of β-arrestin 2 to CXCR2 has been shown to be required for CXCR2 activation (191) and has been reported to be decreased in brain tissue of elderly humans (223). Alternatively, previous reports have demonstrated that high levels of KC promote CXCR2 receptor desensitization and impaired chemotaxis (25). Thus, it is plausible that the early peak in KC levels observed in aged mice acts to attenuate neutrophil recruitment despite bacterial infection in our aged animals. These data extend our findings in our wound infection model, and suggest that neutrophil chemotaxis is perturbed in aged mice.

Aging studies in aseptic wound models have shown that systemic G-CSF enhances neutrophil recruitment and reduces time to wound resolution in aged animals (49). While the role of neutrophils in aseptic wound healing has been questioned (65, 140), patient populations that are at increased risk for infectious complications, such as aging or immunocompromised subjects, demonstrate a necessity for neutrophil recruitment during wound repair (Brubaker *et. al* 2012, manuscript submitted).

Previously, we demonstrated that neutrophils from aged mice exhibit a migratory defect to the murine neutrophil CXCR2 ligand KC *in vitro* and *in vivo* (60). Moreover, we have observed neutrophil chemokine levels to be similar or elevated in wound homogenates and enhanced expression of CXCR2 on circulating neutrophils in aged mice (Brubaker *et. al* 2012, manuscript submitted). Together, these data suggest that age-associated alterations in the downstream CXCR2 signaling cascade may contribute to reduced neutrophil recruitment. G-CSF has been shown to mediate neutrophil migration to macrophage-inflammatory protein-2 (MIP-2), another CXCR2 ligand, via STAT3 mediated induction of CXCR2 expression and regulation of CXCR2 signaling (224). Additionally, *in vivo* G-CSF has been shown to restore *ex vivo* neutrophil chemotaxis in aged rats (200) and following systemic trauma (225). Given these data, it is reasonable to suggest that enhancing neutrophil recruitment in aged mice may reduce bacterial colonization and wound closure. In the study by Nishio *et al*., G-CSF may not only be enhancing circulating neutrophil numbers but may aid in overcoming an age-related migratory defect by enhancing CXCR2 mediated signaling. Based on those data, we attempted to modulated neutrophil recruitment in aged mice via local, *s.c*. G-CSF administration. We demonstrated a beneficial effect of G-CSF treatment on resolution of infection and wound healing in aged mice following *S. aureus* wound infection. These reductions in our primary outcomes were associated with enhanced neutrophil recruitment to the wound site in aged mice following treatment with G-CSF at days 3 and 7. G-CSF treatment did not affect wound macrophage or T cell numbers at days 3 and 7 as compared to saline treated young and aged mice. While the precise mechanism remains unclear, we postulate that restoration of neutrophil recruitment in our model may

be through a G-CSF mediated restitution of CXCR2-directed signaling and chemotaxis in aged mice (**Figure 30**). G-CSF may act to stabilize CXCR2 signaling in aged animals by enhancing downstream factors such as STAT3 and Rap1 (26, 28, 224). Together this would promote upregulation and clustering of integrins, including LFA-1 and VLA-4, which interact with ICAM-1 and VCAM-1, respectively, on activated endothelium (26). Potentially, increased interactions between these molecules could enhance neutrophil firm adhesion and diapedesis, allowing increased neutrophil recruitment to sites of injury and infection in aged animals. To our knowledge, this is the first study to demonstrate the differential effects of G-CSF as a therapeutic modality in the setting of wound infection with advanced aged. Future work needs to be conducted to elucidate the precise mechanism by with G-CSF exerts these age-dependent effects.

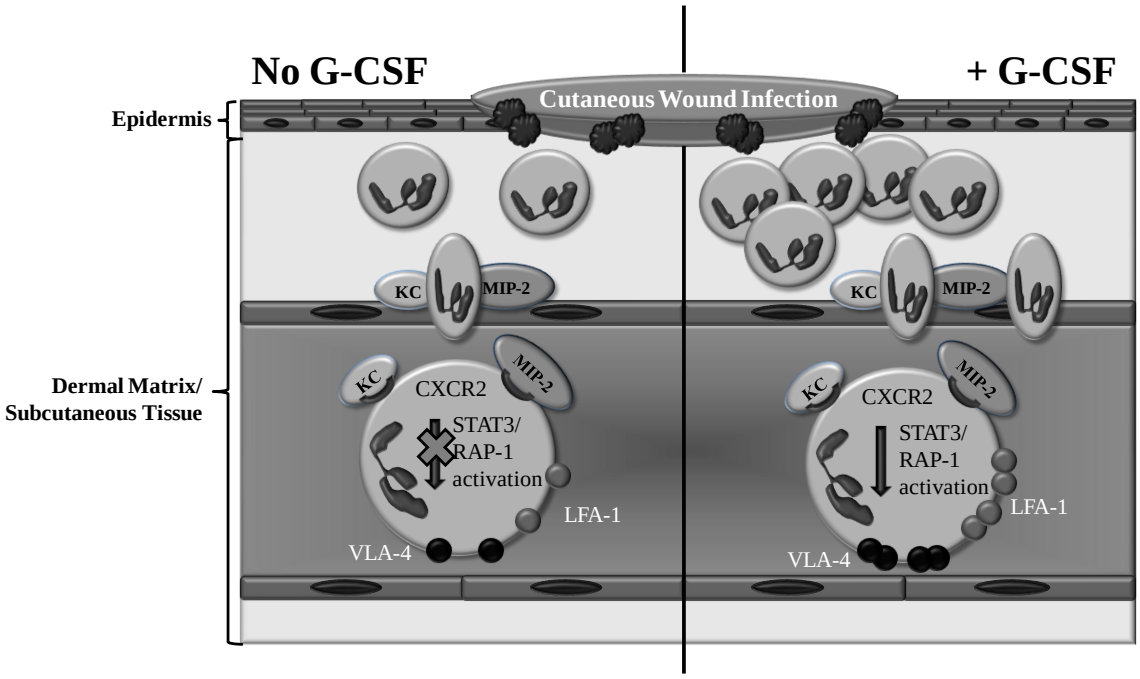


Figure 30. Proposed mechanism: G-CSF treatment restores age-dependent differences in neutrophil recruitment by enhancing signaling involved in neutrophil chemotaxis. We postulate that G-CSF acts to stabilize signaling downstream of CXCR2. Potentially, G-CSF may act to enhance STAT3 or Rap1 activation, increasing upregulation and clustering of integrins that facilitate firm adhesion to the activated endothelium. Conceptually, this would promote elevated adhesion and transmigration of neutrophils to the site of injury and infection. Further studies are needed to determine if this is the mechanism by which G-CSF exerts age-dependent effects in our model.

G-CSF is a known modulator of granulopoiesis and hematopoietic stem cell mobilization as well as a regulator of neutrophil bone marrow emigration, survival, chemotaxis and phagocytosis (226-229). Currently, G-CSF (Filagrastim/PEG-Filagrastim) is indicated for patients with congenital or iatrogenic neutropenias (230) and for donors prior to hematopoietic stem cell transplantation (231). Pre-clinical and clinical trials are underway to investigate the therapeutic potential of G-CSF in amyotrophic lateral sclerosis (232, 233), acute liver failure/alcoholic hepatitis (234), myocardial infarction (235), peripheral arterial disease (236) and diabetic foot infections (237, 238). Alongside our study, these reports provide novel indications for the expanded use of G-CSF as therapeutic interventions.

The studies using G-CSF in the setting of diabetic wound infection provide an interesting point of comparison to the data presented in this manuscript. Cruiciana *et al.* reviewed five randomized controlled trials with a total of 167 patients that added G-CSF to treatment regimens for patients with diabetic foot infections. Though one study reported a shorter time to resolution of infection and intravenous antibiotic use (239), the meta-analysis revealed that addition of G-CSF did not decrease the risk of infectious complications or enhance wound closure, but it did reduce the relative risk of lower limb surgical interventions and the hospital length of stay (237). Similar to advanced aged, neutrophil dysfunction and elevated risk of *S. aureus* infection are associated with diabetes (240). However, the studies above do not suggest a strong therapeutic benefit for G-CSF in diabetic patients. Diabetes is also associated with peripheral arterial disease (PAD) that may inhibit additional facets of wound healing which may not be modulated by G-CSF. Though not evaluated in our studies, aging is associated with alterations in

angiogenesis of local vasculature following cutaneous wound injury (63); however, this differs from the more extensive vascular compromise associated with diabetic PAD. Moreover, studies in db/db mouse models have actually demonstrated elevated neutrophil recruitment to sites of *S. aureus* infection in db/db mice (240). This is in contrast to our model, in which reduced neutrophil accumulation in aged mice was associated with delayed bacterial clearance and wound closure.

Other studies do support a therapeutic benefit for G-CSF in prevention of infectious complications (241, 242). In patients undergoing esophagectomy for esophageal cancer, perioperative, *s.c.* G-CSF improved neutrophil effector functions and elevated circulating leukocyte counts (241). Infection rates and postoperative death were also reduced in the treatment groups as compared to historical controls (241). In a case report, daily G-CSF treatment in a neutropenic patient that developed a non-healing pharyngocutaneous fistula following radical laryngectomy promoted closure of the fistula where previous aggressive wound therapy failed (242). These papers support the use of G-CSF in infectious wound care settings in neutropenic patients. While no peripheral neutropenia was noted in our aged animals prior to G-CSF treatment, we did observe a local reduction in neutrophil numbers at the wound infection site in saline-treated aged mice that was restored following G-CSF administration.

As bacterial strains, in particular *S. aureus*, continually evade our current antibiotic treatments, understanding how advanced age impairs the host immune response may allow us to target immunomodulatory mechanisms that will improve outcomes following cutaneous wound injury in elderly patients. While future work needs to be conducted to elucidate the mechanism by which G-CSF exerts these age-dependent

effects, our data highlights a putative indication for G-CSF treatment within the wound care setting in the elderly patient population as well as patients with local or systemic neutropenia.

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VITA

Aleah L. Brubaker was born on April 9, 1984 in Chicago, Illinois to Linda and Warren Brubaker. Aleah received her secondary education at Oak Park River Forest High School in Oak Park, Illinois. In 2002, Aleah attended Pepperdine University and graduated with a BS in Physiological Psychology in 2006. After graduation, Aleah spent a year working as a teacher for the Kaplan MCAT preparatory course. During this time, she also worked in the laboratory of Dr. Jawed Fareed characterizing low molecular weight heparins and potential neutralizing molecules. Under the mentorship of Dr. Fareed, Aleah was awarded the Julius Jacobson Award for best oral basic science presentation by the International Union of Angiology.

In August of 2007, Aleah matriculated into the MD/PhD program at Loyola University Stritch School of Medicine. In the summer of 2009, she joined Dr. Elizabeth J. Kovacs' laboratory. Under Dr. Kovacs' mentorship, Aleah developed a project examining the effects of aging on the innate immune response to *Staphylococcus aureus* wound infection. From 2010-2011, Aleah was awarded a pre-doctoral fellowship from the Immunology and Aging Program training grant, under the directorship of Dr. Pamela L. Witte. From 2009-2012, Aleah served on the Graduate Student Council as the first-year graduate student representative, GSC co-President and GSC-LUMC council student representative. Aleah will be returning to her complete her third and fourth year medical school clerkships after which she will continue on to residency training.